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Tesi di Laurea Specialistica

Study on the Genetic Determinism of the Resistance of *Medicago truncatula* to *Verticillium albo-atrum*

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Preface

This work was done in the SP2 Lab (Symbiose et Pathologie des Plantes), INP-ENSAT (Université de Toulouse, France), under the supervision of Prof. Laurent Gentzbittel and Dr. Cécile Ben.

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ABSTRACT

Verticillium albo-atrum is a fungus responsible for monocyclic vascular wilt disease in a large amount of species, including economically important crops. It causes a disease known as Verticillium wilt and is one of the most important pathogens in alfalfa.

Medicago truncatula is a spontaneous species in the Mediterranean basin, showing a great biodiversity. It is considered as the model Legume plant, thanks to its autogamy, its short time of reproduction, its diploidy and its small genome. *M. truncatula* has a syntenic relationship with other Legume plants, especially with *M. sativa*, and thus *M. truncatula* can serve as a surrogate for cloning the counterparts of many economically important genes in alfalfa. *M. truncatula* shows a quantitative resistance to *V. albo-atrum*.

Seven different *M. truncatula* spontaneous lines were selected and crossed to obtain Recombinant Inbred Lines (RILs) populations. In the SP2 Lab (Symbiose et Pathologie des Plantes, INP-ENSAT, Université de Toulouse, France) a major Quantitative Trait Locus (QTL) involved in the control of *M. truncatula* resistance to *V. albo-atrum* was detected on chromosome 7 in the RILs population LR5 (resulted from the cross between the most extreme parental lines).

Through the phenotypic and genotypic characterization of the RILs, the QTL confidence interval in the population LR5 was reduced and a QTL was detected in another RILs population, the LR4.

Successful responses to these studies will lead to the identification of few candidate genes for the resistance of *M. truncatula* to *V. albo-atrum*.

At the same time, some analyses were carried out on some *V. albo-atrum* strains that had been genetically modified by the insertion of a gene coding for the GFP protein. This protein shows a green fluorescence: the fungi that express GFP are clearly visible at the fluorescence microscope.

The virulence of these strains was evaluated, in order to use them for the study of the fungus colonization on a cytological level.

RIASSUNTO

Verticillium albo-atrum è un fungo responsabile di una malattia vascolare, nota come "Verticillosi", in un gran numero di specie vegetali, incluse specie coltivate economicamente importanti. In particolare, esso è uno dei più pericolosi patogeni dell'erba medica.
Medicago truncatula è una specie spontanea del bacino del Mediterraneo, caratterizzata da una notevole biodiversità. E' considerata come la Leguminosa modello, grazie alla sua autogamia, al ciclo riproduttivo breve, alla sua diploidia e al suo piccolo genoma. *M. truncatula* ha una relazione sintenica con le altre Leguminose, soprattutto con *M. sativa*; di conseguenza *M. truncatula* può essere utile per la ricerca e il clonaggio degli omologhi di molti geni economicamente importanti nell'erba medica. *M. truncatula* manifesta una resistenza quantitativa nei confronti di *V. albo-atrum*.
Sette diverse varietà spontanee di *M. truncatula* sono state selezionate e incrociate, ottenendo delle popolazioni di *Recombinant Inbred Lines* (RILs). Nel Laboratorio SP2 (Symbiose et Pathologie des Plantes, INP-ENSAT, Université de Toulouse, France) un *Quantitative Trait Locus* (QTL) maggiore per il controllo della resistenza di *M. truncatula* a *V. albo-atrum* è stato identificato sul cromosoma 7 nella popolazione di RILs LR5 (risultante dall'incrocio tra le due linee parentali più estreme).
Tramite la caratterizzazione fenotipica e genotipica delle RILs, è stato ridotto l'intervallo di confidenza in cui è localizzato il QTL nella popolazione LR5 ed individuato un QTL in un'altra popolazione di RILs, la LR4.
Risultati positivi a questa ricerca condurranno all'identificazione di pochi geni candidati per la determinazione della resistenza di *M. truncatula* a *V. albo-atrum*.

Contemporaneamente, sono state condotte alcune analisi su dei ceppi di *V. albo-atrum* che erano stati geneticamente modificati per l'inserzione di un gene codificante per la proteina GFP. Questa proteina emette una fluorescenza verde: i funghi che esprimono la GFP sono chiaramente visibili al microscopio a fluorescenza.
E' stata valutata la virulenza di questi ceppi, che potranno essere utilizzati per lo studio del meccanismo di colonizzazione del fungo a livello cellulare.

INTRODUCTION

The research in the SP2 Lab (Symbiose et Pathologie des Plantes), ENSAT, is mainly centred on the plant *Medicago truncatula* (family *Fabaceae*) and its interactions with symbiotic and pathogen microorganisms, such as *Verticillium albo-atrum*.

Legume plants hold a great agronomic and economic importance. Botanically named *Fabaceae*, this family includes a great number of species, especially herbaceous plants, some used for human nutrition, such as peas (*Pisum sativum*), beans (*Phaseolus vulgaris*) and soy (*Glicine max*), while others for forage, such as alfalfa (*Medicago sativa*) and clovers (*Trifolium* spp.). They are widespread all over the world, but particularly found in mild-to-cold areas. Furthermore, legume plants are particularly valuable as crops because they do not require fertilizer and even enrich the nitrogen content of soil, thanks to their ability of establishing a symbiosis with nitrogen-fixing bacteria. For this reason, they contribute to the sustainability of agricultural ecosystems (Shengming Yang et al., 2008).
Alfalfa, known as the "Queen of Forages", is the world's most important and widely grown forage legume. Its production is negatively impacted by damaging pests and pathogens (Shengming Yang et al., 2008). Amongst these, the *Verticillium* spp., that causes a disease known as Verticillium wilt, is one of the most important pathogens in alfalfa, particularly in Europe, in North America and in the north of Japan. In the USA and Canada yield losses due to the infection with *Verticillium* spp. may reach 50% (Hu GS et al., 2005). *V. dahliae* and *V. albo-atrum*, are responsible for monocyclic vascular wilt disease in over 200 dicotyledonous species, including economically important crops (Pegg and Brady, 2002; Fradin and Thomma, 2006). Several studies have indicated that resistance to Verticillium wilt increases both alfalfa yield (Huang et al., 1994; Viands et al., 1992) and crop profitability (Smith et al., 1995) when disease is present.
The pathogen, that colonizes the vascular system of host plant, cannot be reached by many fungicides, and few fungicides exist to cure plants once they are infected (Emilie Fradin, 2009). The only effective control measure is soil fumigation, which is expensive and has harmful environmental effects (Rowe et al., 1987; Fradin and Thomma, 2006). An improved understanding of genetic and molecular mechanisms underlying host defence will offer novel tools to develop resistant alfalfa cultivars, thus providing an efficient and environmentally sound strategy to control alfalfa diseases (Shengming Yang et al., 2008).

THE PATHOSYSTEM SUBJECT OF THE STUDY

Medicago truncatula

Cultivated alfalfa is autotetraploid (2n = 4x = 32) and out-crossing, making it recalcitrant to genetic analysis, while its diploid relative *Medicago truncatula* is a comparatively simple genetic and genomic system (Cook, 1999). *M. truncatula* is a spontaneous species in the Mediterranean basin, showing a great biodiversity. It is considered as the model Legume plant (Barker et al., 1990): its autogamy, its short time of reproduction (6 months), its diploidy (2n = 2x = 16) and its small genome (500 Mpb) make it a model to simplify the studies about the genetic mechanism of Legume plants responses to abiotic and biotic agents. Nowadays, there are a lot of data and genomic and molecular tools concerning this species, such as EST (Expressed Sequence Tag) libraries, RILs populations, genetic and physical maps and mutants collections, and the sequencing of its genome is in progress.

M. truncatula has not a great importance from an agronomic point of view, but it has a syntenic relationship with other Legume plants, especially with *M. sativa* (Fig. 1). The two species share conserved genome structure and content (Choi et al., 2004), and thus *M. truncatula* can serve as a surrogate for cloning the counterparts of many economically important genes in alfalfa (Shengming Yang et al., 2008).

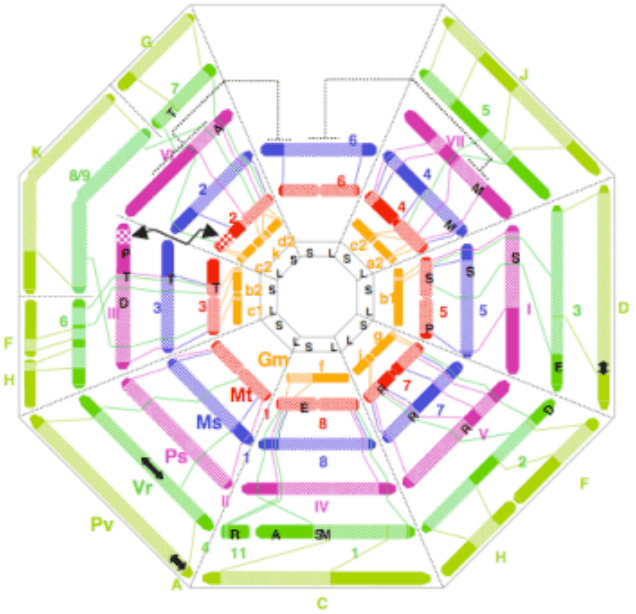


Figure 1: Macrosynteny among six legume genomes. *M. sativa* in blue, *M. truncatula* in red.

To focus on symbiotic plant-microbe interactions, significant efforts have taken advantage of *M. truncatula* as a model system to characterize legume-pathogen interactions (Tivoli et al., 2006). Meaningfully, most alfalfa pathogens are also pathogens of *M. truncatula*, such as the leaf fungus *Colletotrichum trifolii* (Torregrosa et al., 2004) and *Phoma medicaginis* (Ellwood et al., 2006), the root fungus *Fusarium oxysporum* f.sp. *medicaginis* and *Verticillium* spp., and the bacteria *Ralstonia solanacearum* and *Pseudomonas syringae* (Bozso et al., 2009).

Verticillium albo-atrum

Triggered by root exudates, *V. albo-atrum* microsclerotia germinate in the soil and penetrate the roots through the root tip or via wounds and sites of lateral root formation. After crossing the root endodermis, the fungus enters the xylem and produces conidia that are transported by the water stream throughout the plant. Once they have senesced, tissues become colonized; produced microsclerotia are released in the soil during the decomposition of plant materials (Emilie Fradin et al., 2009). The fungus overwinters as mycelium in perennial hosts, plant debris and vegetative propagative parts, and is able to survive in the soil for a long time.

Symptoms in infected plants include chlorosis and desiccation in leaves, and the plant eventually wilts and dies (Leath and Pennypacker, 1990). A transversal section of the roots shows a browning of the vascular tissues (Fig.2).

Figure 2: Necrotic vessels in alfalfa infected by *Verticillium* spp.

There are several strains of *V. albo-atrum*, and they are considerably different as far as the virulence and host range are concerned. In any case, all the *V. albo-atrum* strains develop at a medium temperature of 25°C (Christen and French, 1982; Heale 1985); the infection usually takes place in the middle of the summer.

V. albo-atrum is spread through seeds, infected vegetable debris, root contacts, garden tools, pollen, wind and water as well as through insects carrying the pathogen.

PLANT-PATHOGEN INTERACTIONS AND THE GENETICS OF RESISTANCE

The plant-pathogen interaction can lead to resistance phenomena. There are two different types of resistance:

- The qualitative resistance, also called race-specific, monogenic or vertical resistance, which is based on a gene-for-gene relationship (Hammond-Kosack and Parker, 2003). The resistance phenomenon is the result of an incompatible reaction, due to the interaction between the plant resistance gene and the pathogen virulence gene when they are both in the dominant form (R-Avr). If at least one of these genes is in the recessive form (R-avr, r-Avr and r-avr), the compatible reaction takes place and the plants shows its susceptibility to the pathogen (Tab.1). Such resistance differentiates clearly between races of a pathogen, as it is effective against specific races of the pathogen and ineffective against others (Agrios, 2005).

	Resistance or susceptibility genes in the plants	
Virulence or avirulence genes in the pathogen	R (resistant) dominant	r (susceptible) recessive
Avr (avirulent) dominant	AvrR (-)	Avrr (+)
avr (virulent) recessive	avrR (+)	avrr (+)

Table 1: Quadratic check of gene combinations and disease reaction types in a host-pathogen system in which the gene-for-gene concept for one gene operates. The minus sign indicates incompatible (resistant) reactions and therefore no infection; the plus sign indicates compatible (susceptible) reactions and therefore the infection develops.

- The quantitative resistance, also called partial, polygenic or horizontal resistance, which is controlled by several genes. All plants have a certain, but not always equal, level of possibly unspecific resistance that is effective against each of their pathogens

(Agrios, 2005). In other words, it appears in a range of tolerances depending on the alleles combination of the genes involved in the resistance.

Varieties with qualitative resistance generally show complete resistance to a specific pathogen under most environmental conditions, but a single or few mutations in the pathogen may produce a new race that may infect the previously resistant variety. On the contrary, varieties with quantitative resistance are less stable and may vary in their reaction to the pathogen under different environmental conditions, but a pathogen will have to undergo many more mutations to completely break down the resistance of the host.

Monogenic resistance is easy to manipulate in a breeding program, and therefore is often preferred to polygenic resistance; however, the research for quantitative resistance leads to a more durable protection of the plant.

The highly effective approach for studying the complex form of the plant disease polygenic resistance is the Quantitative Trait Locus (QTL) mapping, which is somewhat easier than the Association Mapping.

In several plant species, including alfalfa, cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), strawberry (*Fragaria vesca*), sunflower (*Helianthus annuus*) and tomato (*Solanum lycopersicum*), sources of genetic resistance to *Verticillium* spp. have been described (Schaible et al., 1951; Lynch et al., 1997; Bae et al., 2008). For example, Simko et al. (2004) detected three significant QTLs for the resistance to the fungus in a potato diploid population.

Most of these crop species contain genes for tolerance or partial resistance, but not complete resistance to the fungus (Fradin and Thomma, 2006). The tomato is an exception in which resistance to race 1 of *Verticillium* isolates is conferred by a single dominant locus that was introduced in cultivated varieties in the 1950s (Schaible et al., 1951; Diwnan et al, 1999) and that is still carried by most commercial tomato varieties. This locus, known as *Ve*, comprises two closely linked inversely oriented genes, *Ve1* and *Ve2*; Fradin et al. (2009) demonstrated that solely *Ve1* determines the resistance of the tomato toward race 1 strains of *Verticillium*.

The successful inter-species transfer of R genes has been reported in many plant species (Thilmony et al., 1995; Seo et al, 2006). While there are examples that *R* genes are functional when transferred across family boundaries, they generally exhibit restricted taxonomic functionality (Tai et al., 1999), conferring a resistance response only in closely related species within a family. Given the close polygenic relationship between *M.*

truncatula and alfalfa, functional *R* gene transfer in *Medicago* should not be a challenge (Shengming Yang et al., 2008). Shengming Yang et al. (2008) demonstrated that the *M. truncatula R* gene *RCT1* confers broad-spectrum anthracnose resistance (*C. trifolii*) when transferred into susceptible cultivated alfalfa plants. They previously mapped the *RCT1* locus to *M. truncatula*, and then they cloned three candidate genes of the locus and transferred them in susceptible alfalfa plants; in the end, they were able to detect the *RCT1* gene amongst the candidates.

QTL: Quantitative Trait Loci

QTLs are loci controlling part of the phenotypic variation of a quantitative character; their physical position on the chromosome is definite and invariable. These loci can identify regions on the chromosome where there are major genes (genes particularly important in character determination) or group of genes involved in the determination of the same quantitative character and closely placed on the chromosome.

The molecular markers are polymorphic genomic loci that could be detected through the use of specific probes or primers. They are suitable for measuring the genetic variability of a population, designing the chromosomes maps and making the selection (Barcaccia and Falcinelli, 2005).

A good marker should be:

- Polymorphic (i.e. it should have different alleles);
- Co-dominant (that allows to distinguish the heterozygous from the homozygous);
- Simple and not expensive to be studied;
- Reproducible and transferable to other genotypes of the same species.

Molecular markers carrying all these features would be ideal, but they are very rare to find. The DNA molecular markers are the best ones, because they are almost unlimited in number, they are independent of environmental conditions and they don't vary with the age of the plant or the analysed organ.

There are different kinds of DNA molecular markers, but, as I have dealt with the SSR and SNP, I will describe only these two types.

The Single Sequence Repeats (SSR) are microsatellites: these sequences usually have a variable length amongst the individuals of a species; therefore they could be used as markers. To check the differences on the SSRs level, it is sufficient to design primers on

the flanking region of the microsatellite, and then amplify it through PCR. The following electrophoresis will make the different length of the amplified fragments visible.

The Single Nucleotide Polymorphism (SNP) markers are based on the difference on a single nucleotide level. To detect these markers the sequencing of the genome is needed; therefore, they are the most precise molecular markers, but they are also the most expensive.

The QTL analyse is based on the comparison between the genotype and the phenotype of recombinant lines derived from the inbreeding between parental lines that are phenotypically opposite concerning the interested character.

Recombinant Inbred Lines (RILs)

It is one of the main types of mapping populations. RILs are formed by crossing two inbred strains followed by repeated selfing to create some new inbred lines whose genomes are mosaics of the parental genomes (Fig 3).

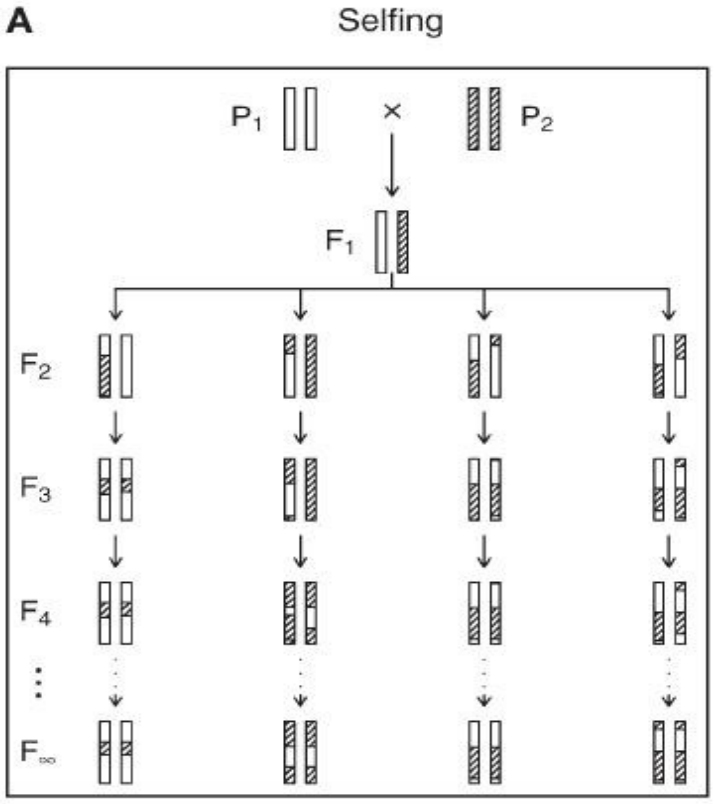


Figure 3: A RIL population is obtained by two parental lines that are homozygous (one dominant and one recessive) for the interesting character. When they are inbreeded there is recombination, so the F1 is the generation with the highest heterozygosity possible. Then, going on with 6-7-8 selfings, at the F7-F8 generation there will be a lot of individuals, different from each other, but with higher homozygosity, each of them with some chromosome traits of both the parents.

Strategies to detect QTLs

First of all, the individuals of the RILs population must be phenotypically and genotypically characterized, with the help of molecular markers.

Then, the basic principle is to divide the population in the genotypic classes based on the markers (for example AA, AB and BB) and then determine if there is correlation between the genotype and the phenotype. If there were, that position would carry on a QTL.

To test the hypothesis of the presence of a QTL in a RILs population, the LOD score must be calculated. The LOD (Logarithm of the odds ratio) score is a likelihood ratio, and it is calculated as follows:

$$LOD = \log_{10}[V(a_1,d_1)/V(a_0,d_0)]$$

where $V(a_1,d_1)$ is the likelihood function under the hypothesis of the presence of a QTL whose parameters are a_1 and d_1, calculated by linear regression, and $V(a_0,d_0)$ is the likelihood function under the hypothesis of the absence of QTL (Lander et Botstein, 1989). For example, a LOD score = 2 means that the presence of the QTL in the determined position is 100 times more probable than its absence.

There is appropriate software that calculates the LOD score along the entire chromosomes and gives the results in a graphical way (Fig.4). Where there is a peak, it means that there is a major significant relation between that position on the chromosome and the quantitative character: to sum up, in that zone there is a gene (or a group of genes) which controls that character. The software establishes also a significant threshold (named LOD threshold) through statistical analyses based on the input data: only the peaks over that threshold would be considered corresponding to a QTL. The QTL confidence interval is defined, by convention, as the chromosome trait that corresponds to the LOD score of the peak ± 1.

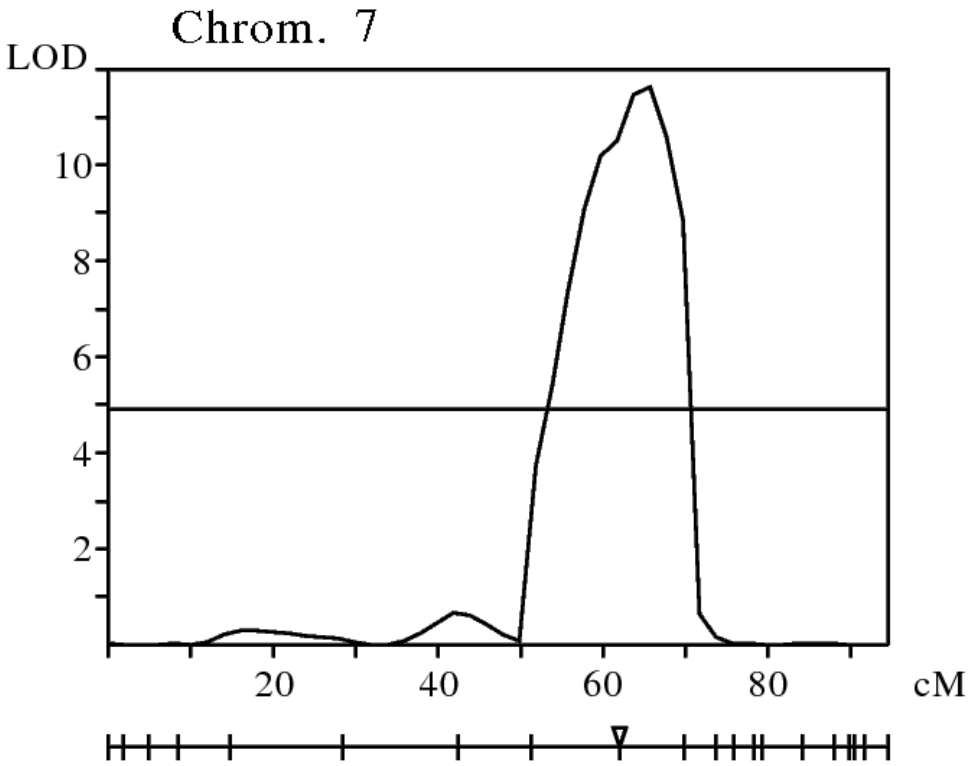


Figure 4: Graphical representation of the LOD scores on the chromosome 7 of *Medicago truncatula* for the resistance to *Verticillium albo-atrum*; on the x-axes the markers along the chromosome and the lengths in cM, on the y-axes the LOD scores. The line corresponding to a LOD score of 4, 8 is the LOD threshold.

PREVIOUS RESEARCH IN THE SP2 LAB

In the SP2 Lab, two different approaches were developed for the research on the pathosystem *M. Truncatula* / *V. Albo-atrum:*

- the study of the determination of the genetic mechanism causing the differential response resistance/susceptibility of the plant to the pathogen, that is the main subject of my project;
- the study of the *V. albo-atrum* mechanism of infection on a cytological level.

GENETIC STUDIES

The research began with the selection of seven different *M. truncatula* spontaneous lines (Jemalong A17, F83005.5, TN1.11, TN1.21, DZA45.5, DZA315.16 and Jemalong A20), that were crossed to obtain RILs populations (Fig. 5).

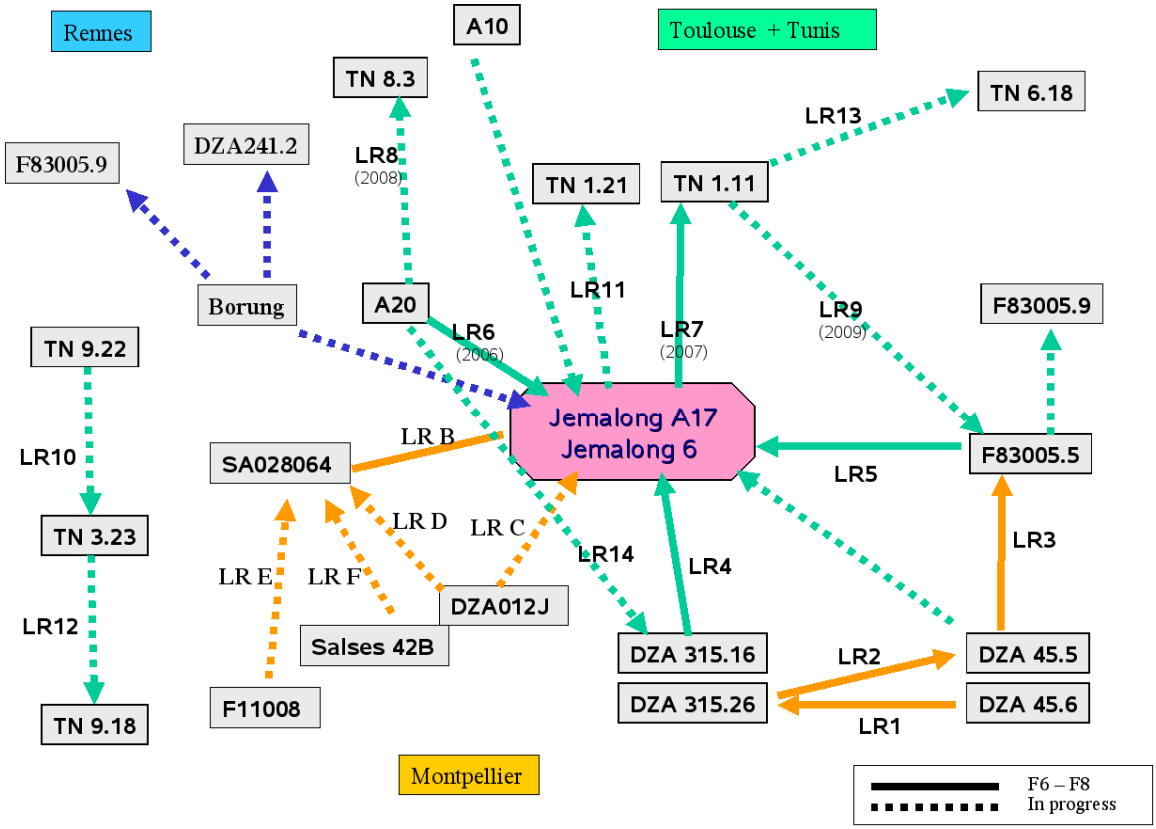


Figure 5: RILs populations (LR) obtained by the inbreeding between the 7 parental lines of *Medicago truncatula*. The full arrows mean that the RILs population are fixed (the lines are homozygous) and mapped; the dotted arrows mean that the RILs population are not fixed yet. This work was done in collaboration with the INRA located in Montpellier, France.

First of all, the response to the inoculation with *V. albo-atrum* was characterized in the seven parental lines, in order to determine the most contrasting amongst them. The lines Jemalong A17 (following called A17) and F83005.5 (following called F83) showed the most opposite responses, and they are, in particular, respectively resistant and highly susceptible to the *V. albo-atrum* infection. The other lines gave intermediate responses between A17 and F83; a susceptibility scale was designed with six of the seven analysed lines (the ones that were significatively different) (Fig.6).

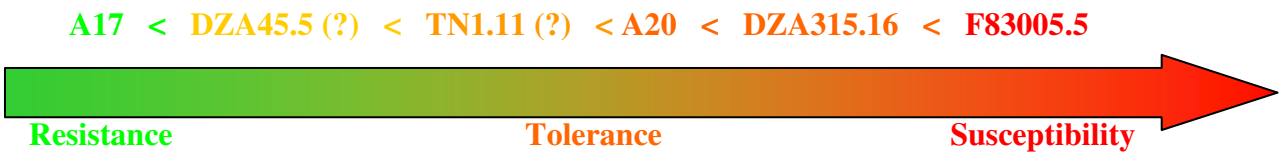


Figure 6: Six *M. truncatula* parental lines in a susceptibility scale.

The data obtained by these experiments showed that the *M. truncatula* resistance to *V. albo-atrum is* a quantitative resistance, and it is, therefore, linked to one or more QTL.
Based on the classification of the lines, some possible inbreeding was detected for the study of the genetic determinism of the resistance:

LR5: A17 x **F83005.5**

LR6: A17 x **A20**

LR4: A17 x **DZA315.16**

LR1: DZA315.16 x **DZA45.5 (?)**

Beginning with the population LR5, resulting from the cross between the most extreme lines (A17 x F83), the tolerance level of the RILs was evaluated, and this work has enabled to detect a major QTL, on chromosome 7, involved in the control of the *V. albo-atrum* resistance. The QTL confidence interval is 6 cM long and this area includes 31 BACs and two gaps.

CYTOLOGICAL STUDIES

To better understand the infection mechanism of *V. albo-atrum* and their relationship with the different tolerance degree of *Medicago* lines, cytological studies were carried out on in the SP2 Lab. The *V. albo-atrum* wild strains were genetically modified by the insertion

(through the ATMT technique: *Agrobacteriun tumefaciens* Mediated Transformation) of a gene coding for the GFP protein. This protein shows a green fluorescence: the fungi that express GFP are clearly visible at the fluorescence microscope. In this way, a description of the colonization of the plant by the pathogen would be possible.

Four different fluorescent strains were obtained in the SP2 Lab: *C3A1 b', C3A1 a', C3D2 c'* and *C3A1 b*. Their level of fluorescence was tested, giving the following results:

C3A1 b Bte.4	+++
C3A1 a' Bte.2	++
C3A1 b' Bte.4	++++
C3D2 c' Bte.3	+

where the number of + represents the brightness of the fluorescence.

GOALS OF THE PROJECT

My work in the SP2 Lab was centred on the study of the genetic determinism of the model Legume plant *M. truncatula* resistance to the fungus *V. albo-atrum*. In this framework, I had two complementary goals:

- the reduction of the QTL confidence interval in the RILs population LR5, obtained by the inbreeding of the two most contrasting parental lines, A17 and F83;
- the detection of a QTL in the RILs population LR4, obtained by the inbreeding of A17 and DZA315.16 (that is the most susceptible parental line to *V. albo-atrum* after F83).

Successful responses to these studies would lead to the identification of few candidate genes for the resistance of *M. truncatula* to *V. albo-atrum*.

At the same time, I carried out some analyses on the *V. albo-atrum*-GFP strains, and in particular I made an evaluation of their virulence on the parental lines A17 and F83. This test is necessary before going ahead with the study of the fungus colonization at a cytological level using these strains.

MATERIALS AND METHODS

GENETIC STUDIES OF THE RESISTANCE OF MEDICAGO TRUNCATULA TO VERTICILLIUM ALBO-ATRUM

1. Reduction of the QTL confidence interval in the LR5 population

The RILs population that was analysed is the LR5, obtained by the inbreeding between A17 and F83, respectively the most resistant and the most susceptible lines amongst all the parental lines. For this population a major QTL was known, so I chose to analyse the lines that showed recombination in the QTL zone, to try to reduce its confidence interval.
The steps involved in the work are the following:

- Phenotyping: the lines are inoculated with *Verticillium albo-atrum*, and the development of the symptoms is observed and scored, with a conventional index, for 27 days.
- Detection of new markers: new SSR (Single Sequence Repeats) markers are detected in the QTL zone, and the flanking primers are designed.
- Genotyping: the genomic DNA of the phenotyped plants is extracted and determined through PCR + electrophoresis, to check for some polymorphisms of the new markers amongst the RILs.

Phenotyping

I analysed the RILs of the LR5 population that showed a recombination in the QTL zone; in all, I had 53 lines at the F8 or F9 generation, plus the two parental lines A17 and F83, as control. For most of the lines I tested 6-8 plants, but in some cases I had fewer plants, or even only one, because of problems in germination.
The seeds, obtained by the pods, are scarified with sandpaper and then placed in little Petri dishes on a piece of blotting paper imbibed with sterile water. The dishes are kept at 4°C in obscurity for 2-3 days, to overcome seed dormancy, and then for 24 h at 25°C (or for 48 h at 20°C) to let them germinate.

The germinated seeds are transplanted in *Jiffy pots* (*Jiffy*, Lyon, France) and then placed in little greenhouses in the *phytotron*, with a day/night cycle of 16/8 h, with a temperature of 25/20°C and a RH (Relative Humidity) of 75%.

V. albo-atrum is grown on PDA medium (Potato-Dextrose Agar, *Difco*™: Potato starch + Dextrose) in Petri dishes, at a temperature of 24°C and in obscurity (12-15 days).
I used the *V. albo-atrum V32* strain, the only wild strain available today in France.

The plants are inoculated when they are 10 days old.
The solution of the spores for inoculation is prepared by making a suspension of 10^6 spores/mL of sterile water (concentration verified with the "Malassez cell").
To make the spores absorption uniform in all the plants, the roots are clipped by cutting off a layer of about 1 cm at the bottom of each *Jiffy pot*. Then the *Jiffies* are dipped into the spores' solution for 30 minutes.
At the end the *Jiffy* are placed on plates containing commercial potting soil and transferred in a *phytotron* with a day/night cycle of 16/8 h, with a temperature of 20/20°C and a RH of 57,9%.

The development of the symptoms is checked 2 days/week for 27 days from the inoculation. The first symptoms appear about 7 days after the inoculation.
For the evaluation of the degree of the symptomatology, I used a disease severity index rating from 0 to 4 (Fig.7):

- 0 = no symptoms;
- 1 = the first leaf is chlorotic;
- 2 = the first two leaves wilt;
- 3 = the entire plant wilts;
- 4 = the plant is dead.

<u>Detection of new markers</u>

The detected QTL for the resistance to *V. albo-atrum* is located on the chromosome 7 of *M. truncatula*; this zone includes 31 BACs and two gaps. The SSR markers for the first and the last BACs (AC146554 and AC135311, respectively) are available.

Figure 7: The disease severity index used for the evaluation of the symptoms development in *Jiffy pots*. 0 = no symptoms; 1 = chlorosis of the first leaf; 2 = wilt of the first leaves; 3 = wilt of the plant; 4 = death.

In order to refine the molecular map in the QTL confidence interval, I detected eleven new markers, located on one BAC every two or three (all the information about the *M. truncatula* genome is available on the web site www.medicago.org).

To detect the SSR markers I used the software SSRIT (www.gramene.org/db/markers/ssrtool): it needs, as input, the sequence on which detecting the SSRs (the BAC), the maximum length of the repeated motif (dimer, trimer or tetramer) and the number of repetitions of this motif (I chose a minimum of 5), and it gives, as output, a list of all the SSRs and their characteristics (i.e. the motif, the number of repeats, where the SSR starts and ends and its length). I selected the best SSRs from this list, choosing the microsatellites with the higher number of repeats, the dimers and the TA motifs.

Once the SSRs were found, in order to use them as markers I had to design the primers on their flanking regions, to be able to amplify them by PCR. To do this, I used the software Primer3 (http://frodo.wi.mit.edu/primer3/): it needs, as input, the sequence including the microsatellite, the PCR product size ranges (150-300 pb) and general primer picking conditions (size, Tm, etc.), and it gives, as output, a list of 5 pairs of primers (Forward and Reverse) with their characteristics. The fundamental features that a pair of primers should have are:

- The F and the R-primers should have the same length, with a maximum difference of 1 nucleotide;
- The Tm (melting temperature) should differ of 1°C at most;
- The matching probabilities inter and intra primers should be lower than 5.00.

In table 2 the list of the SSR markers that I detected and of which I designed the flanking primers is shown.

The last two are markers that I found on the web site www.medicago.org.

The primer's sequence specificity was checked through a Blast analysis by using the software CViT-Blast (www.medicago.org) in the *M. truncatula* genome with default parameters.

The polymorphism of the new markers must be tested on the parental lines (A17, F83 and DZA315.16) before being used for the genotyping of the RILs: for each line, two repetitions of PCR + electrophoresis tests were done.

BAC ACCESSION	*N°MTIC*	*Rep. motif*	*Primer F*	*Primer R*
AC123899	1421	(TA)26	GAGATGTGTAACGCCCCAAT	CCCCCAAAACTCCAATTATTC
AC148481	1422	(AT)22	TTGCTCAACTCGTCCAAAAA	TGGCAAATGTCGCTGAATAC
AC150384	1423	(AT)28	TTTCCACAACTTGGGCTATG	CCCCCGCTATCTTTTTGTAA
AC155886	1424	(AT)23	TTCGAATTCCGGAGAGAAAA	GAAAAAGGTGTTGGTCGCTAA
AC155890	1425	(AT)15 CA	CAACGGACTCAGAGAGAGAA	TGGTGTCAACGGATCCTAACT
AC162277	1426	(TA)30 AA	CAACACGCCAAACTTAAATAG	CACCCGCTTAGTTTTTAGAATG
AC169173	1427	(AT)16	CAGGGTTCTGATGGTGGTCT	CACGTGATGTCTTTGGTTGG
AC169178	1428	(AT)17	TAGTTGCCTGCATTTGCTGT	CGAAAAGATCGCCAAGGATA
AC197893	1429	(TA)10	CCTGTTGAGTTGGAGCATTG	GGAAAAGGCATGCACACATA
AC232697	1430	(TA)27	ATGACGCCTGATTGAAAGGA	CAGCACGGGTGGAAAGTCTA
CU151875	1431	(AAT)5	GGCTTGATGTACTTTTCGTTCC	TTCAATCCTTCGTGAACCAGA
AC149809	1432	(TC)25	TTCGCTACCACCATCCTCTC	AGTGGTTGAACGAATCTCCG
AC146784	1433	(AT)18	GCCGAATATGCGAGCTTTT	TGGCTCACCTTCCACTTCA

Table 2: The detected SSR markers: the BAC on which they are located, their identification name, the microsatellite sequence with the number of repetitions and the primers sequences.

Genotyping

At the end of the symptoms survey, I went ahead with the genotyping of the plants. The genotyping consists of a PCR of the phenotyped LR5 RILs with the new primers, followed by a gel electrophoresis. I used the primers for markers Mtic 518, 1045 (already available in the Lab), 1423, 1424, 1425, 1426 and 1431.

To extract the genomic DNA from a plant, three young leaves are harvested and placed in a well of a plate with 96 wells of 2,5 mL each; then, 2 glass beads are added in each well. The leaves are dried at 65°C for one night, and then grinded with the "beads grinder" (1 min at maximum power). 500 µL of extraction buffer (2 mL Tris-HCl 1 M + 0.5 mL NaCl 5 M + 0,5 mL EDTA 0,5 M + sterile DNase-free water for 10 mL of buffer) are added to each well, the plate is shaken and then centrifuged for 20 min at 4000 rpm. The supernatant from each well is transferred to a new plate and centrifuged 20 min at 4000 rpm; again, the supernatant is transferred to a new plate, and 300 µL of isopropanol are added to each well. The plate is shaken and centrifuged 30 min at 4000 rpm. The pellet is dried 5 min under

the laminar flow hood and then re-suspended in 100 µL of DNase-free water. The plate should be kept at -20°C.

I extracted the DNA from one plant/line, except for the lines that were still heterozygous in the QTL zone: for these lines, I extracted the DNA from all the plants and analysed them separately. Then, I made the PCR + electrophoresis with all the markers mentioned above.

The PCR mixtures for 1 µL of genomic DNA (diluted to 10 in sterile DNase-free water) consists of: 2,5 µL of 10X PCR Buffer (*Interchim, France*), 1,25 µL of $MgCl_2$ 50 mM (*Interchim, France*), 1 µL of each dNTP 1,25 mM (*Promega, Madison*, WI, USA), 1 µL of each primers (*Invitrogen™, France*), suspended in sterile water (25 ng/µL), 0,25 µL of *Taq*-Polymerase and 14 µL of sterile water, for a total of 25 µL. The ordinary reaction conditions are: 94°C for 4 minutes, followed by 38 cycles of 94°C for 30 seconds, 55°C for 30 sec and 72°C for 30 sec, followed by 72°C for 6 min. The amplified products are separated by gel electrophoresis on 3,5% Agarose (2,6% UltraPure Agarose + 0,9% "Low melting" Agarose) in 1XTAE buffer, and stained with ethidium bromide.

The reading of the electrophoresis gel was useful to design a table of the genotype of each line in the markers' zone, giving the A genotype to the lines that were equal to A17 (the female line, by convention) and B genotype to the lines that were equal to F83 (the male line).

2. QTL detection in the LR4 population

The RILs population LR4 is obtained by the inbreeding between A17 and DZA315.16. It is the most interesting population after LR5, because DZA315.16 immediately follows F83 for the susceptibility to *V. albo-atrum* (Fig. 6).

The steps of the experiment are the following:

- Phenotyping: the method is the same as that used for the LR5 population;
- QTL detection: based on the results of the phenotyping, and using the *M. truncatula* linkage map of markers available in the SP2 Lab, I checked the LR4 population for one or more QTL for the resistance to *V. albo-atrum*.

Phenotyping

I analysed 113 RILs of the LR4 population, dividing them in two groups: a first group with 61 lines, and a second with 68; 16 lines were tested twice. All the lines were at the F7 or F8 generation. In each group, I added the parental lines of the population, A17 and DZA315.16, as well as F83. I tested 6-8 plants per line.

The procedure is exactly the same as that used for the phenotyping of LR5.

QTL detection

The data obtained by the phenotyping of the 138 RILs were used for the detection of the QTL. To do that, I used the software PLABQTL: it needs, as input, the linkage map of markers and the phenotypic data of the lines. The outputs, obtained by the program calculations for each trait and chromosome, which were interesting for me are:

- estimates of parameters such as additive effects, dominance effects and R^2;
- a final simultaneous fit with all detected QTL.

B. THE EVALUATION OF THE VIRULENCE OF THE VERTICILLIUM ALBO-ATRUM-GFP STRAINS

I analysed the virulence of the *V. albo-atrum*-GFP strains by inoculating the plants in hydroponic culture and checking the development of the symptoms, in comparison with the *wild type* (*V32*)-inoculated plants and mock-inoculated plants.
At the end of the symptoms survey, I observed the roots of the plant through the fluorescence microscope.

Phenotyping

The germinated seeds of A17 and F83 (the proceeding for the germination is the same explained above) are placed on boxes full of a nutritive solution (*Farheus* medium – 1 mL $MgSO_4x7H_2O$ + 1 mL $KHPO_4$ + 2 mL Na_2HPO_4 + 1 mL $FeC_6H_5O_7$ – added with $CaCl_2$), and then incubated at 24°C.

Some plants are inoculated after 10 days, others after 4 days. For each *V. albo-atrum* strain, a solution of spores is prepared by making a suspension of 10^6 spores/mL of sterile water. The roots of the plant, previously cut at about 6 cm, are dipped in the solutions for 60 minutes; some A17 and F83 plants, at both ages, are dipped in sterile water, as control. Then, the plants are placed once again on the boxes with the *Farheus* medium and incubated at 20°C (the optimal temperature for the *V. albo-atrum* growth).

The first symptoms appear about 7 days after the inoculation, and from this moment on they must be checked everyday, because in the hydroponic culture they develop very quickly. For the 10-day plants, the indexes are the same as the inoculation in *Jiffy*, while for the 4-day plants I used another scale:

- 0 = no symptoms;
- 1 = the cotyledons are chlorotic;
- 2 = the cotyledons are necrotic;
- 3 = the first leaf is chlorotic;
- 4 = the first leaf is necrotic;
- 5 = the plant is dead.

I checked the symptoms until 16 dpi.

Microscope observation

Because of the inserted gene for the GFP expression, the transgenic *V. albo-atrum* strains can be observed with an optical microscope.

At the end of phenotyping, I harvested the plants for the microscopic observation: I picked one plant of A17 and one of F83 for each *V. albo-atrum*-GFP strains, and one plant from the control; I collected only the plant inoculated after 10 days, because almost all the others were dead.

The work consisted of the following steps:

1. Inclusion in agarose gel: a portion of 2-3 cm of the root is cut near the crown and then included in a cylinder of "low melting" agarose gel at 5%. I prepared an inclusion in agarose gel for each collected plant;
2. Cutting with the vibratom: the vibratom allows making very thin sections of the samples that must be observed. I prepared about 8 sections of 110 μm of thickness for each sample and transferred them on slides in sterile water;

3. Microscope observation: I observed the samples with the optical microscope through a blue filter at 10X and 20X enlargement. The blue filter allows one to see all the emissions with a higher wavelength than the blue, i.e. the red, the yellow and the green of the GFP.

RESULTS AND DISCUSSIONS

1. Reduction of the QTL confidence interval in the LR5 population

Phenotyping of the LR5 RILs

The results of the phenotyping are shown in the graphic (Fig. 8): the lines, parental ones included, are placed in growing order according to the degree of susceptibility to *V. albo-atrum*.

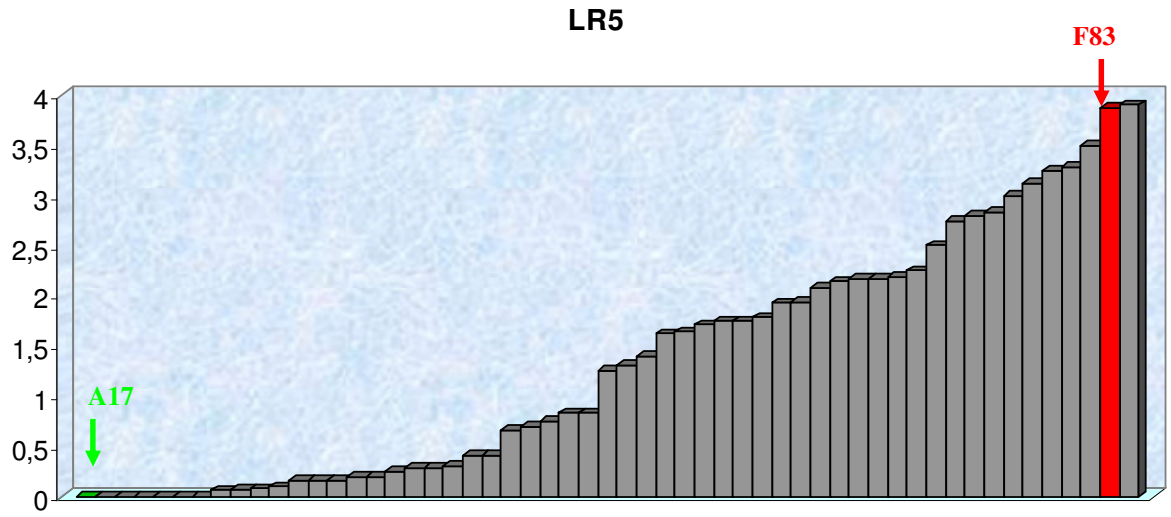


Figure 8: Histogram showing the results of the phenotyping of LR5. On the y-axes the score of the disease; each bar represents a RIL that recombines in the QTL confidence interval. The red bar is referred to F83 (susceptible) and the green to A17 (resistant).

The large variability of the resistance is clearly visible, and I can also notice the presence of a trasgressive line, i.e. a line that has a response to the infection more extreme than the parental line; in particular, line LR5 F9 104 seems more susceptible than F83. This is an ordinary effect of the quantitative resistance in a population of RILs. In any case, a statistical analysis is necessary to confirm the difference between the two lines.

It is also important to underline the variability between the different plants of a line. To show that, I have designed the curves representing the disease kinetics for each plant tested for each line, and the mean of all the plants of a line (Annexe I).

Moreover, I have compared the responses of my work with the responses of an older one with the same RILs (Fig. 9). The correlation coefficient is of about 0,67: the more this coefficient is near to 1, the higher the correlation.

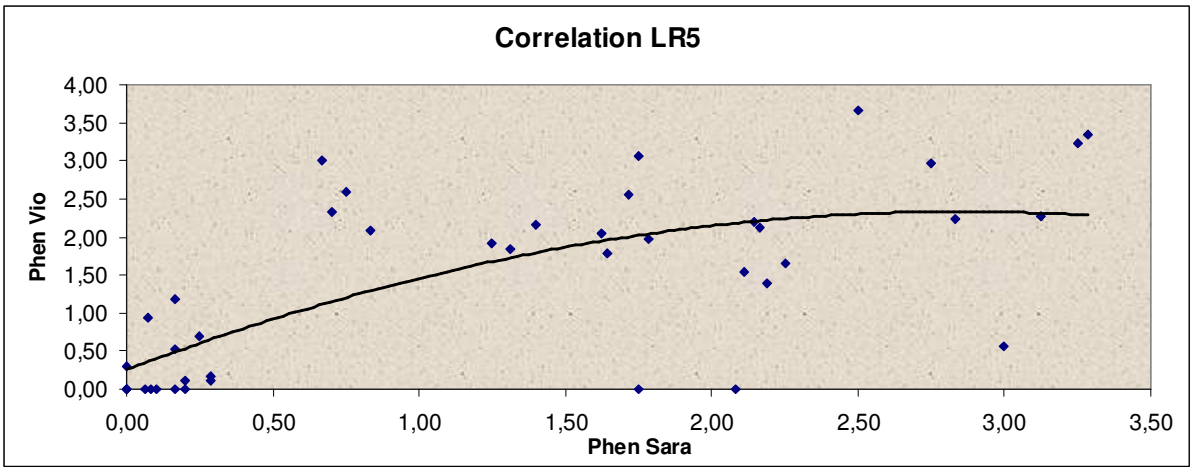


Figure 9: Graphic showing the correlation between two different phenotyping of LR5: the data used are the mean scores for each line at 27 dpi. On the x-axes the responses of my phenotyping; on the y-axes an older one.

The coefficient that I calculated is quite distant from 1, and it means that the environment influence is quite high in the determination of this phenotype. In fact, the quantity of spores in the solution for the inoculation could not be exactly the same in two different assays, as long as the time during which the roots are dipped in it. Moreover, because of the great number of plants tested at the same time, they are not all exposed at the same conditions of humidity and temperature: in fact, some plants dehydrated before others, according to the position occupied in the *phytotron*, and this has, obviously, an influence on the stress degree of the plant. Eventually, the method of the disease severity index for the evaluation of the symptoms development is not too accurate, and, in particular, it is quite subjective; it means that, if the survey is done by two different people, there will be a different margin of error in the assignment of a score.

Test of the new SSR markers

Tests by PCR + electrophoresis showed a polymorphism between A17 and F83 for the markers Mtic 1423, 1424, 1425, 1426, 1427, 1430, 1431 and 1432; they are also all co-dominants, except for Mtic 1427 and 1430, which seem not to amplify F83. Therefore, they can be used for the genotyping of LR5. Moreover, there are also good perspectives for

the genotyping of LR4: in fact, the marker Mtic 1423 shows a clear polymorphism between the three parental lines, and the markers Mtic 1425, 1431 and 1433 are clearly polymorphic for A17 and DZA315.16.

The other markers either gave feeble bands, or showed a non-specific amplification, or did not work at all; so, they should be tested again, optimizing the PCR (i.e. increasing the Tm).

Where there is no size polymorphism, the PCR products could be sequenced, to find out some polymorphisms at the level of single nucleotides (SNP markers).

Table 3 sums up the responses of the test for each marker.

Mtic	*BAC*	*Responses*	*Following analyses*
1421	AC123899	It did not work	TEST AGAIN
1422	AC148481	No size polymorphism	SEQUENCING
1423	AC150384	Polymorphism among the 3 lines, co-dominance	AVAILABLE
1424	AC155886	Polymorphism among the 3 lines, co-dominance	TEST AGAIN
1425	AC155890	Polymorphism A17-F83 and A17-DZA	SEQUENCING
1426	AC162277	Polymorphism A17-F83 and F83-DZA	SEQUENCING
1427	AC169173	Polymorphism, but no co-dominance	TEST AGAIN
1428	AC169178	It did not work	TEST AGAIN
1429	AC197893	No size polymorphism	TEST AGAIN
1430	AC232697	Polymorphism, but no co-dominance	TEST AGAIN
1431	CU151875	Polymorphism A17-F83 and A17-DZA	SEQUENCING
1432	AC149809	Polymorphism A17-F83 and F83-DZA	SEQUENCING
1433	AC146784	Polymorphism, but no co-dominance	TEST AGAIN

Table 3: The responses of the tests of the SSR markers, and the following analyses that should be done.

Genotyping of the phenotyped LR5 RILs

Tables 4 and 5 represent the responses of the genotyping, done by PCR + electrophoresis, of the RILs population LR5 (Fig. 10). The A genotype is referred to the lines that were equal to the female parental line and the B genotype to the lines that were equal to the male parental line; the H is referred to heterozygous.

	LR5 F9 2	LR5 F9 5	LR5 F9 12	LR5 F9 14	LR5 F9 19	LR5 F9 21	LR5 F9 25	LR5 F9 26	LR5 F9 38	LR5 F9 39	LR5 F9 40	LR5 F9 42	LR5 F9 44	LR5 F9 45	LR5 F9 52	LR5 F9 53	LR5 F9 60	LR5 F9 61	LR5 F9 62	LR5 F9 64	LR5 F9 79	LR5 F9 81	LR5 F9 82	LR5 F9 89	LR5 F9 90	LR5 F9 91	LR5 F9 92
Mtic 1045 (MTE85)	H	A	A	B	B	B	B	B	A	B	A	B	B	B	B	A	B	A	A	B	B	A	A	B	A	B	A
Mtic 1431	H	A	A	B	B	B	B	B	H	?	?	B	B	B	H	A	A	A	A	B	?	A	A	B	A	B	A
Mtic 1426	H	?	?	B	B	B	B	B	A	B	A	B	?	B	?	A	B	A	A	B	B	A	?	B	A	?	A
Mtic 1423	H	A	A	B	B	B	B	B	?	B	?	B	B	B	B	A	B	A	A	B	B	?	A	B	A	B	A
Mtic 1425	B	A	A	B	B	B	A	B	A	B	A	B	A	B	B	A	B	?	A	B	B	A	A	B	A	B	A
Mtic 1424	B	?	?	?	?	?	?	?	?	B	?	?	?	?	B	A	B	A	A	B	B	?	?	?	?	?	H
Mtic 518 (MTE126)	?	A/H	A/H	B	B	B	A/H	B	A/H	B	A/H	B	A/H	B	B	A/H	?	A/H	A/H	B	B	A/H	A/H	B	A/H	B	A/H
Phenotyping 1																											
Medium score 27 dpi	2,17	2,17	0,42	2,75	3,00	2,50	0,83	2,83	1,75	3,50	0,06	0,83	1,75	0,70	0,00	0,08	0,00	0,00	0,25	0,67	0,42	2,80	0,10	1,40	0,20	2,08	0,17
n° plants	6	6	6	4	1	6	3	6	6	4	8	6	6	5	4	6	1	4	8	3	6	5	5	5	5	6	6
Phenotyping 2																											
Medium score 27 dpi		2,12	NA	2,98	0,56	3,67	2,08	2,23	0,00		0,00		3,06	2,33	0,29	0,00	0,00	0,00	0,70	3,00		NA	0,00	2,17	0,00	0,00	0,00
n° plants																											

Table 4: Responses of the genotyping, with seven SSR markers, of the phenotyped LR5 lines. The mean scores for each line at 27 dpi of my phenotyping; and of the older one are added.

	LR5 F9 94	LR5 F9 99	LR5 F9 100	LR5 F9 103	LR5 F9 104	LR5 F9 113	LR5 F9 115	LR5 F9 120	LR5 F9 142	LR5 F9 144	LR5 F9 147	LR5 F9 157	LR5 F9 161	LR5 F9 162	LR5 F9 181	LR5 F9 185	LR5 F9 190	LR5 F9 191	LR5 F9 193	LR5 F9 199	LR5 F9 200	LR5 F9 201	LR5 F9 202	LR5 F9 210	LR5 F9 213	LR5 F9 215
Mtic 1045 (MTE85)	A	B	B	B	B	B	B	B	A	B	A	A	A	B	H	?	A	B	A	B	A	B	?	B	H	B
Mtic 1431	A	B	B	B	A	B	B	B	A	B	A	A	A	A	A	A	A	B	A	B	A	B	A	B	H	B
Mtic 1426	A	B	B	A	?	?	B	B	A	B	?	A	?	B	B	A	B	B	?	B	?	B	A	B	?	B
Mtic 1423	A	B	B	B	H	B	B	B	A	B	A	A	A	B	H	H	B	B	A	B	A	B	A	B	A	B
Mtic 1425	B	B	B	B	A	A	B	A	A	B	?	A	?	B	B	A	B	B	A	B	A	B	?	B	A	?
Mtic 1424	B	?	?	?	A	?	?	?	?	?	?	A	?	A	?	?	?	?	?	?	A	B	A	B	A	?
Mtic 518 (MTE126)	B	B	B	A/H	A/H	?	B	A/H	A/H	B	A/H	B	A/H	A/H	A/H	A/H	B	B	A/H	?	A/H	B	A/H	B	A/H	A/H
Phenotyping 1																										
Medium score 27 dpi	0,00	0,75	0,31	2,11	3,90	2,19	0,00	1,25	0,00	1,71	3,29	0,17	1,63	1,31	0,17	1,93	0,20	0,29	3,13	3,25	0,29	2,14	0,07	1,79	1,64	2,25
n° plants	7	6	8	8	5	8	3	6	2	7	7	6	8	8	6	6	5	7	8	6	7	7	7	7	7	8
Phenotyping 2																										
Medium score 27 dpi	0,00	2,59		1,53	2,00	1,38	0,00	1,91		2,56	3,34	0,53	2,05	1,84	1,18		0,12	0,17	2,27	3,24	0,12	2,20	0,94	1,97	1,78	1,66
n° plants																										

Table 5: Continuation of the table above**.**

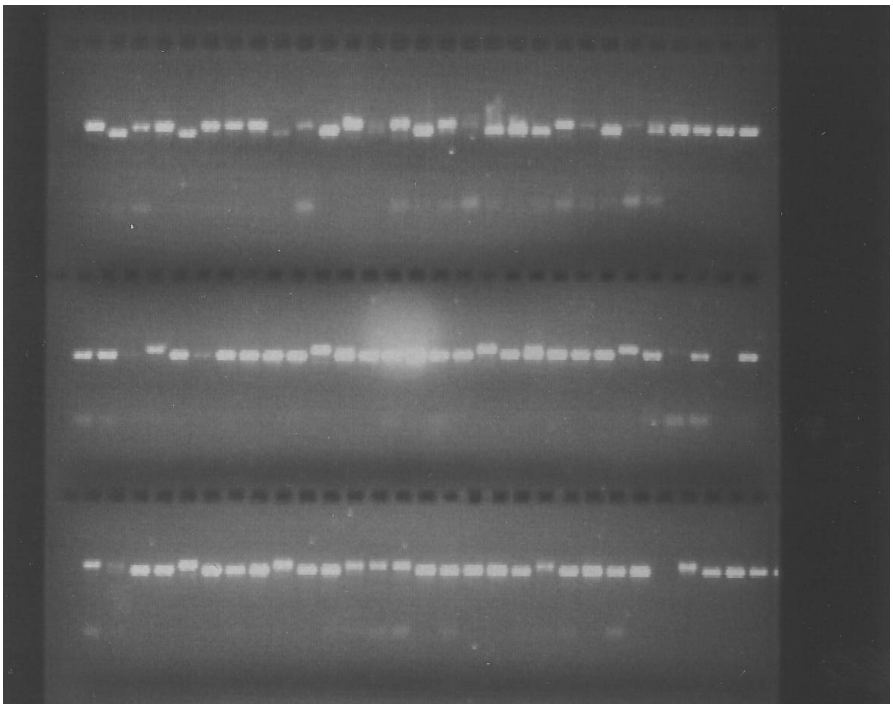


Figure 10: Photo of the gel electrophoresis of the LR5 lines amplified with the marker Mtic 1431.

The Mtic 518 is revealed to be dominant: in both the repetitions that I did with it, the first with a Tm of 55°C and the second of 60°, it did not work with the male line. It means that the B genotypes can be identified (they correspond to the absence of bands), while the discrimination between the A and H genotypes is not possible.

Observing the responses in the tables, I can notice the presence of lines that are still heterozygous in some marker's locus. For these lines, another (or more) selfing generation is needed to fix the genotype.

Even if there is a lot of data missing, due to mistakes in the PCR, problems with the markers or impossibility in reading the gel, observing the lines that recombined in the confidence interval (LR5 F9 25, F9 44, F9 60?, F9 94, F9 103?, F9 104, F9 113, F9 120, F9 157, F9 162?, F9 190) I can underline a major correspondence with the phenotype of the regions over the Mtic 1425 (generally, only the lines with a score under 1 are considered resistant). On the contrary, the genotype of some lines seems not to be related with the phenotype, but for all of them there is missing data: a second analysis of these genotypes could give positive responses.

Finally, even if the responses of the study must be refined, collecting more data for the phenotyping and repeating the PCRs with the new primers, there are strong chances that the QTL were located between the Mtic 1045 and the Mtic 1425.

2. QTL detection in the LR4 population

Phenotyping of the LR4 RILs

The results of the phenotyping are shown in the graphic (Fig. 11).

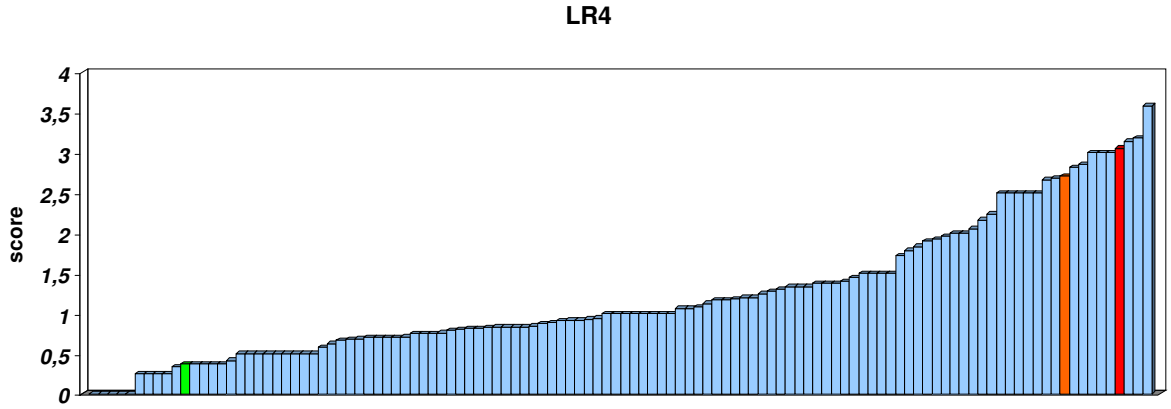


Figure 11: Histogram showing the results of the LR4 phenotyping. On the y-axes the score of the disease; each bar represents a RIL. The orange bar is referred to DZA315.16 and the green to A17, the two parental lines; the red bar is referred to F83, as a comparison.

As for the LR5 population, I can notice the large variability of the resistance and the presence of some trasgressive lines (that must be confirmed with statistical analysis): the lines LR4 F8 8, 49, 51, 69, 141, 140, 135 and 61 have shown an higher susceptibility than DZA315.16 (and the last three are also more susceptible than F83), and the lines LR4 F8 210, 105, 136, 54, 13, 235, 224, 91, 62 and 25 higher resistance than A17.

In Annexe II, the graphics with the curves representing the disease kinetics are reported.

For the 16 lines that I tested twice, I calculated the correlation coefficient, that is of about 0,79. It is higher than the coefficient calculated for LR5, and also in the graphic (Fig. 12) we can clearly see that, in this case, the correlation is higher. However, the available data for calculating the correlation is quite less than the data I had for LR5, and therefore the results are not so reliable. Moreover, I observed an almost uniform difference in the severity of the symptoms between the two repetitions (Fig. 13): almost all the scores of the first repetition are lower than the scores of the second one (again, this must be statistically proved), and it well confirms the importance of the environment in the determination of the phenotype.

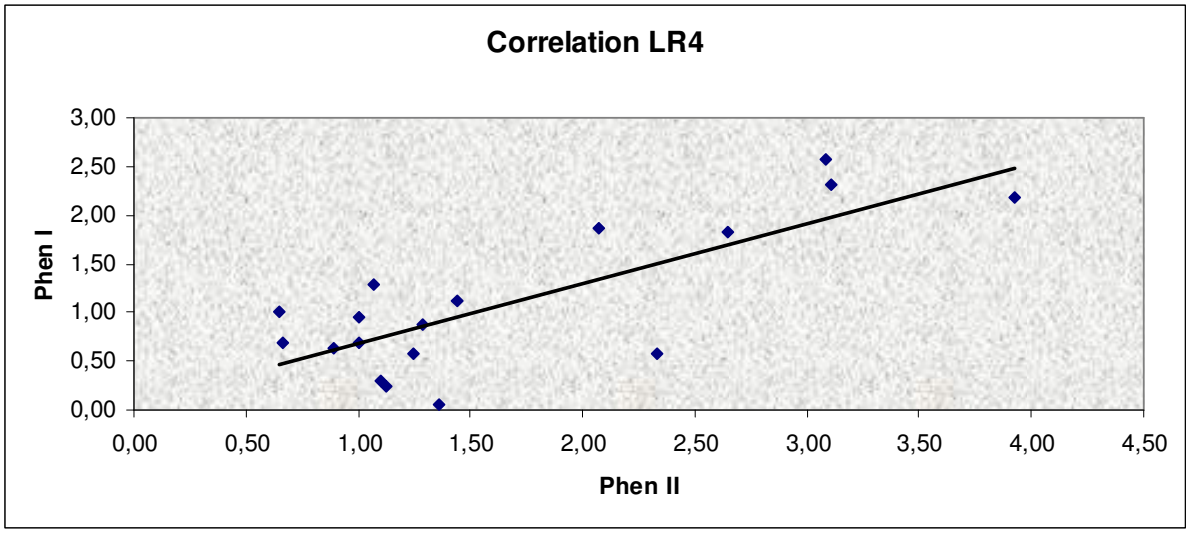


Figure 12: Graphic of the correlation between the two repetitions of 16 lines of LR4: the data used is the mean scores for each line at 27 dpi. On the x-axes, the responses of the second repetition and on the y-axes the first one.

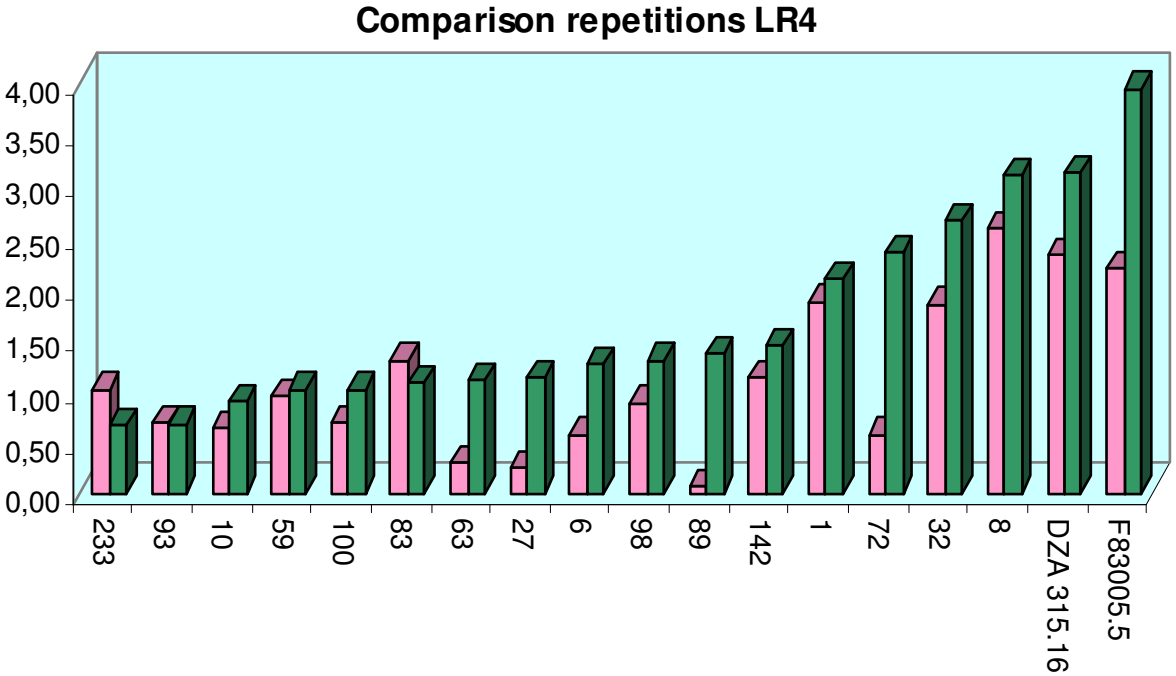


Figure 13: Histogram showing the difference in the severity of the symptoms for the two repetitions of 16 lines of LR4. The pink is referred to the first repetition, the green to the second one.

QTL detection on LR4 population

The QTL detection was done with the means at 27 dpi for the lines of the first group, and then with the means of all the tested lines. A major QTL was found out on the chromosome 7 (Annexe III). In Table 6 the estimated parameters of the QTL, calculated through PlabQTL, of the first group and of all the lines, are shown.

```
LIST OF DETECTED QTL, LODscore >    2.500
---  with new scanning fit  ---
verticilium scan general                Mean27M1  (SIM)
QTL  Chrom.  Pos Left_Mark   Mark.I+Pos cM_n.M. Supp.IV    LOD  R^2%      add
-------------------------------------------------------------------------------
---
1  chrom7   42 MTE73m51   51-  52  11.  10.   34-  52   3.86  26.4    0.535
-------------------------------------------------------------------------------
---

LIST OF DETECTED QTL, LODscore >    2.500
---  with new scanning fit  ---
verticilium scan general                Mean27M1M (SIM)
QTL  Chrom.  Pos Left_Mark   Mark.I+Pos cM_n.M. Supp.IV    LOD  R^2%      add
-------------------------------------------------------------------------------
---
1  chrom7   42 MTE73m51   51-  52  11.  10.   36-  48   8.17  29.2    0.570
-------------------------------------------------------------------------------
---
SUM:  1                                                      29.2
```

Table 6: The lists of all the detected QTLs for the first group of LR4 lines and for all the LR4 lines, and their estimated parameters.

The interesting parameters are:

- `Pos`: the position on the chromosome of the QTL in cM;
- `Left_Mark`: the name of the left flanking marker;
- `Supp.IV`: support interval with a LOD fall of 1.0 (default), expressed as position on the chromosome, in cM. Note: a support interval is only determined for the global QTL peak in a given region, i.e. by ignoring other adjacent peaks in the case of multiple peaks;
- `LOD`: the LOD score of the QTL peak;
- `R^2%`: the coefficient of determination or the percentage of phenotypic variance, which is explained by a putative QTL;
- `add`: estimated additive QTL effects at the location of scanning. The additive effect is half the difference between the genotypic values of the two homozygotes. It is assumed that the second parent carries the favourable alleles for the trait under study. If the second parent is the weaker one, additive effect becomes negative.

The most reliable values are those in the list for all the LR4 lines (the more the lines are phenotyped, the finer is the detection); so, the peak of the detected QTL is at 42 cM from the beginning of the chromosome 7, and the first known marker located on the left is the MTE73. The confidence interval is 12 cM length, that is quite large; the LOD score of the peak is 8.17 (the LOD threshold is at 2,5). The percentage of phenotypic variance explained by the QTL is only 29,2%; the additive QTL effects are of 0,57.

The QTL known for the LR5 population has the peak at 66 cM, and the left flanking marker is the MTE85. The confidence interval is 6 cM length and it begins 14 cM after the end of the confidence interval of the detected QTL for LR4.

These are only the preliminary results, but they are very encouraging for the prosecution of the study. In fact, the QTL detected for LR4 is placed on the same chromosome where the QTL for the LR5 population is located, and they are not so distant (Fig. 14). Further analyses with a huge number of lines for LR4 could give successful responses.

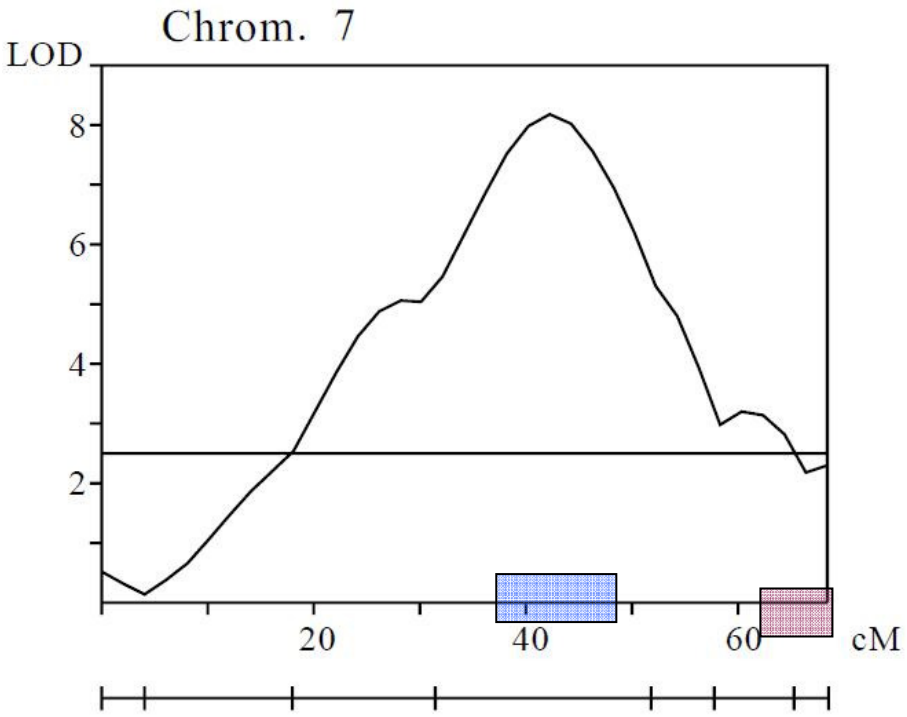


Figure 14: The position of the QTL for the LR4 population on the genetic map of *M. truncatula*. In blue the confidence interval of the QTL for LR4, and in purple the confidence interval of the QTL for LR5.

3. Evaluation of the virulence of the V. albo-atrum-GFP strains

Advantages and disadvantages of the hydroponic culture

Given that the assay with the *V. albo-atrum*-GFP strains was done in hydroponic culture, I am able to make some considerations about this method, comparing it with the *Jiffy-pots*' one.

In hydroponic culture the development of the symptoms is faster; therefore, this method has allowed us to have a sufficient number of responses in half the time. Moreover, the plants in hydroponic culture take up less space than in the *Jiffy-pots* (Fig. 15).

But the hydroponic culture also has its disadvantages. First of all, the A17 line does not bear the hydroponic conditions very much, showing an earlier senescence that, sometimes, ends in death. This is, obviously, a substantial problem for these kinds of assays, because the cause related to the plant suffering becomes difficult to be distinguished. Furthermore, the hydroponic culture is quite far from reality: the plants are weaker than if grown on a solid substrate, and, at the same time, the pathogen is in the best conditions for its development and for its virulent activity.

Figure 15: Difference in the space taken up by hydroponic and *Jiffy-pots* cultures. The photos were taken at the end of phenotyping (27 dpi for the plants in *Jiffy* and 17 dpi for the plants in hydroponic culture).

However, the hydroponic culture is the only method that can be used for these kinds of assays, because it allows observing and picking up the roots of the plants.

Statistical analysis of the responses of the virulence test with the transgenic *V. albo-atrum* strains

Both for A17 and for F83, the plants inoculated after 4 days showed a higher stress; therefore, they cannot be taken into consideration.

In the graphics (Fig 16) the responses of the two lines, A17 and F83, inoculated after 10 days, to the different *V. albo-atrum* strains are represented. The data used to design the graphic are the mean scores at 15 dpi, until which the highest difference was observed between the two lines.

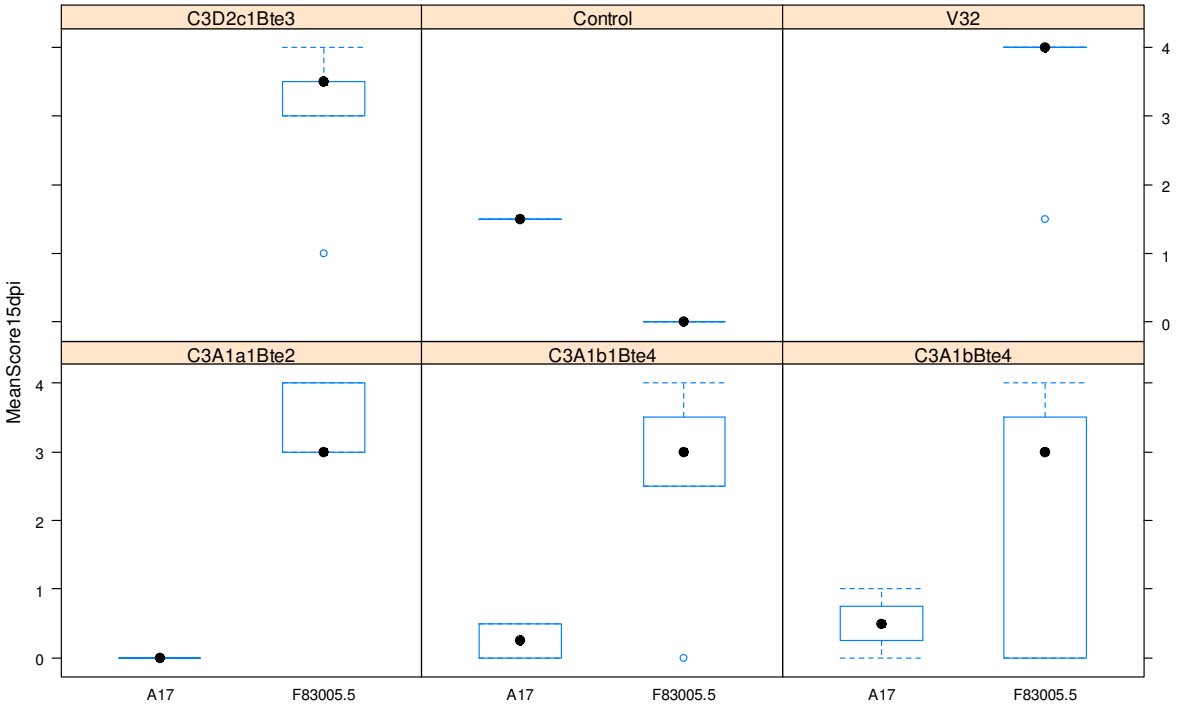


Figure 16: Box plots showing the responses of the virulence test of the *V. albo-atrum*-GFP strains. Each square represents a strain (included the wild strain and the control); on the y-axes the score.

Observing the box plot for the control, the suffering of A17 in hydroponic culture is evident. In any case, the other box plots show that A17 is more resistant than F83 to the strains *C3A1 a', C3A1 b'* and *C3A1 b;* for the other strains (*C3D2 c'* and *V32*) I had no A17 plants. The variability of the lines, in particular of F83, is clear, too.

The responses were statistically analysed through the software R (ANOVA). Because of the lack of some data, the variance analysis was done separately for the line and the fungus strain effect. The graphic below represents the variance due to the strain effect (Fig.17).

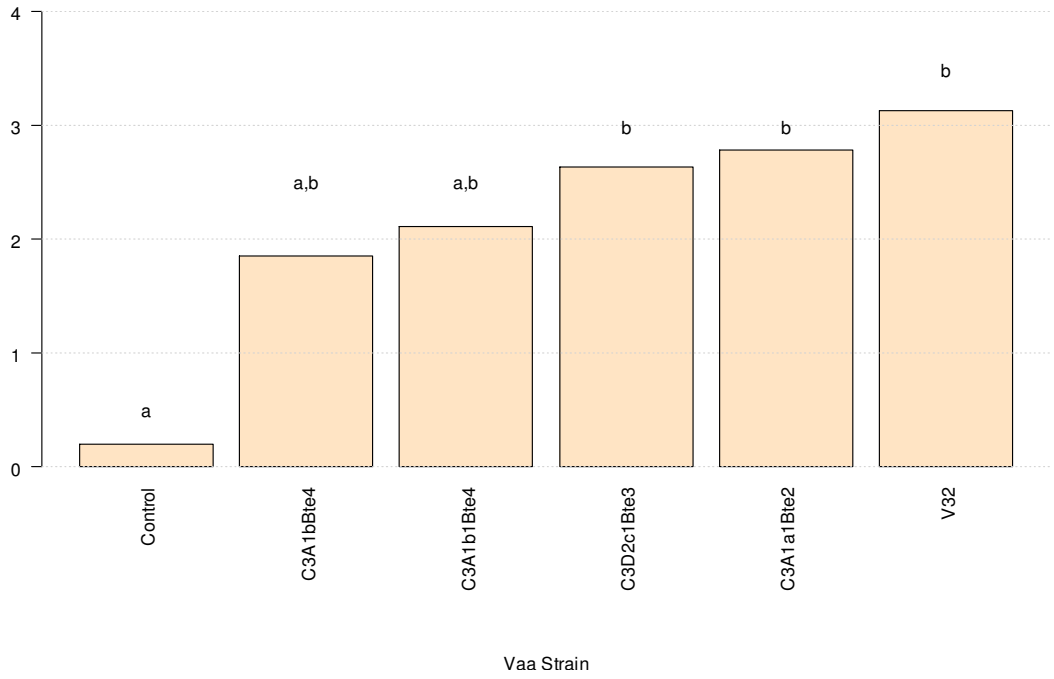


Figure 17: Histogram representing the variance in all the tested plants (both A17 and F83, inoculated after 10 days) due to the strains effect. On the y-axes the scores.

Two strains that are identified by the same letter are not significatively different. So, all the strains, both the transgenic and the wild type, have a similar virulence. Moreover, the two strains *C3A1 b* and *C3A1 b'* seem not to be significatively different from the control. Therefore, the interesting transgenic strains are the *C3D2 c'* and *C3A1 a'*, that are significatively different from the control, but similar to *V32*. This means that they can be used for the study of the colonization pathway of *V. albo-atrum*. Unluckily, they are the least fluorescent strains amongst the four that were tested.

Microscope observation

With the optical microscope through a blue filter it is possible to see all the emissions with a higher wavelength than blue. The root has a natural fluorescence that goes from red (chloroplast) to yellow-pale green (vessels and parenchyma) (Fig. 18), while the GFP has a bright green fluorescence.

Amongst all the observed samples, I found traces of GFP only in one of them (Fig. 19): the mycelium of the *C3A1 b* strain was visible in the vessels of the F83 root, the susceptible line. In all the other samples there were no visible *Verticillium* at all. The hypothetical explanation is that the promoter for the inserted gene that codes for the GFP is

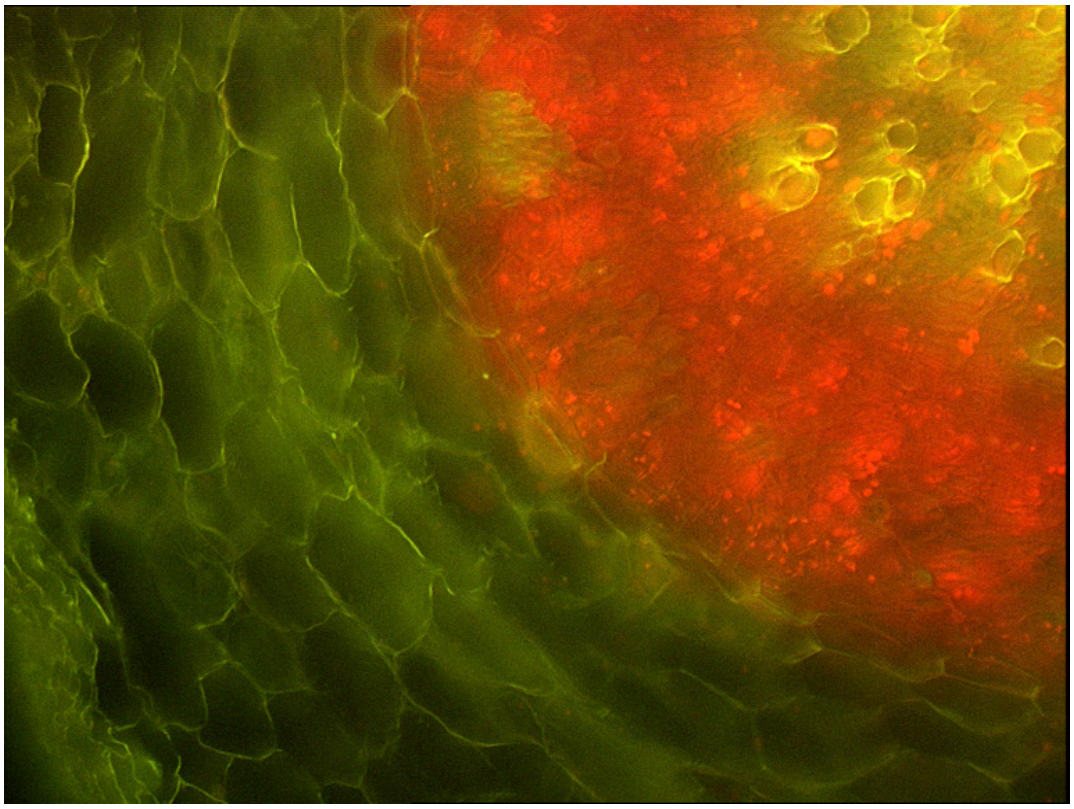


Figure 18: Photo at the optical microscope through a blue filter of a sample of a non-inoculated F83 root.

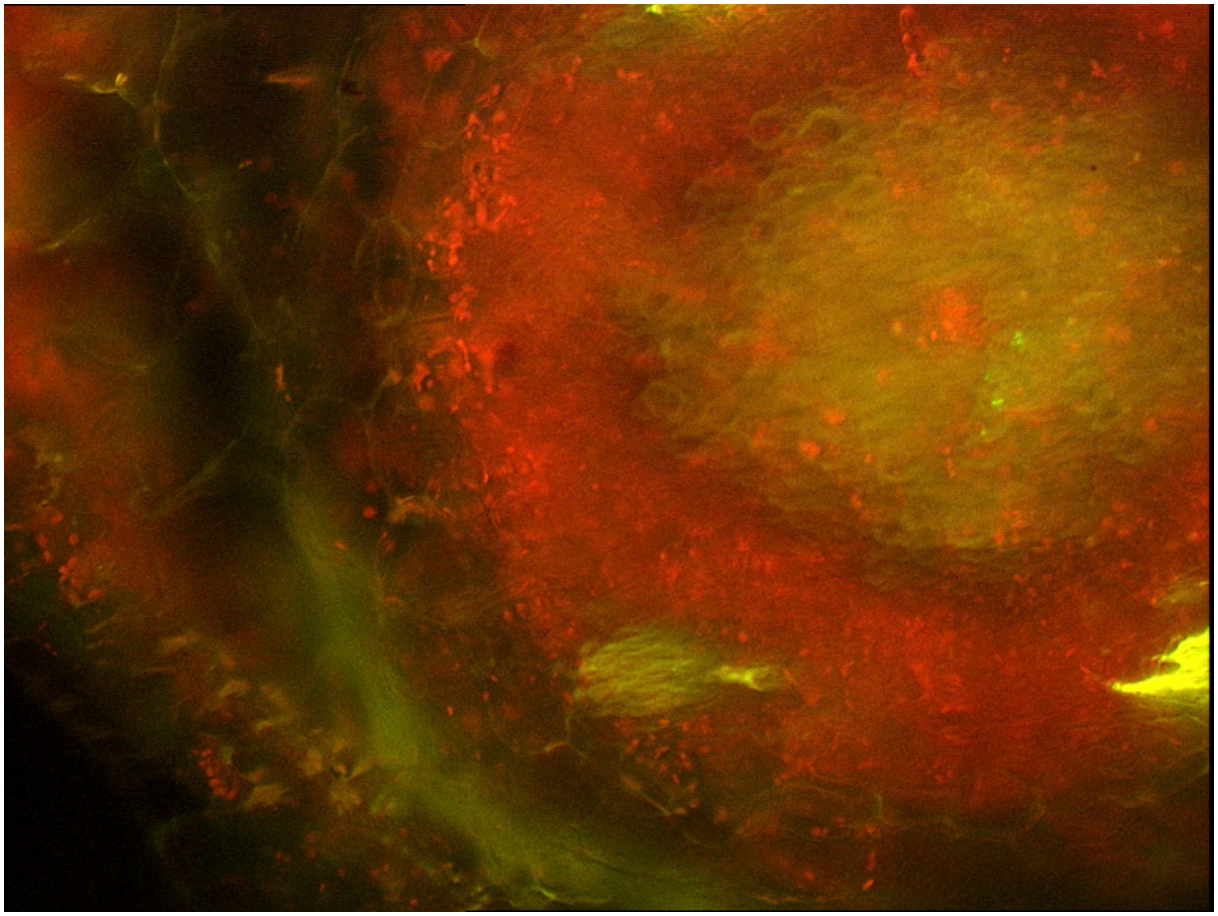


Figure 19: Photo at the optical microscope through a blue filter of a sample of a F83 root inoculated with the *V. albo-atrum C3A1 b*. We can see traces of the mycelium, marked with the GFP, in the vessels.

non-constitutive: it is the glyceraldehyde-3-phosphate dehydrogenase promoter of *Aspergillus nidulans*, which is linked to the glycolysis. R. O'Connell at al. (2004) used the same vector that was used in the SP2 Lab (pBin-GFP-hph vector, including the gene for the GFP, under the control of the promoter mentioned above, and the selection marker for the resistance to the hygromycine B) to transform *Colletotrichum destructivum*, and they observed a bright fluorescence in all actively growing fungal structures produced during the infection; but they made their observation at 55 h after inoculation. Given that I observed the plants at 17 dpi, when the fungus metabolic activities were lower, it is possible that the non-constitutive expression of the GFP was too low to see the fluorescence. However, this hypothesis has not been confirmed yet.

CONCLUSIONS AND PERSPECTIVES

Concerning LR5, additional analyses would be done through the detection of new markers, focussing on the region over the marker Mtic 1425.

At the same time, the QTL detection in the LR4 population should be refined, through the phenotyping of a greater number of RILs and the improvement of the phenotyping method. This could be possible, for example, finding some parameters for taking into account the variations of the environmental conditions. In any case, in the SP2 Lab another phenotyping method is under analysis: the quantification of the fungus in the inoculated plants through Real Time PCR. The development of this method would surely be suitable for the phenotyping of the RILs populations.

An improvement of the assay's methods and a greater number of data would also lead to the discovery of other major or minor QTLs.

Further reductions of the QTL confidence interval could allow the detection of 5-6 candidate genes, that will be analysed to find the one (or more) related to the resistance to *V. albo-atrum*. To do that, there are several approaches, such as the introduction of the candidate gene in the susceptible lines, with the following observation of the symptomatology, or the VIGS (Virus-Induced Gene Silencing).

The functional annotation of the genes included in the QTL zone would help in the detection of the candidate genes.

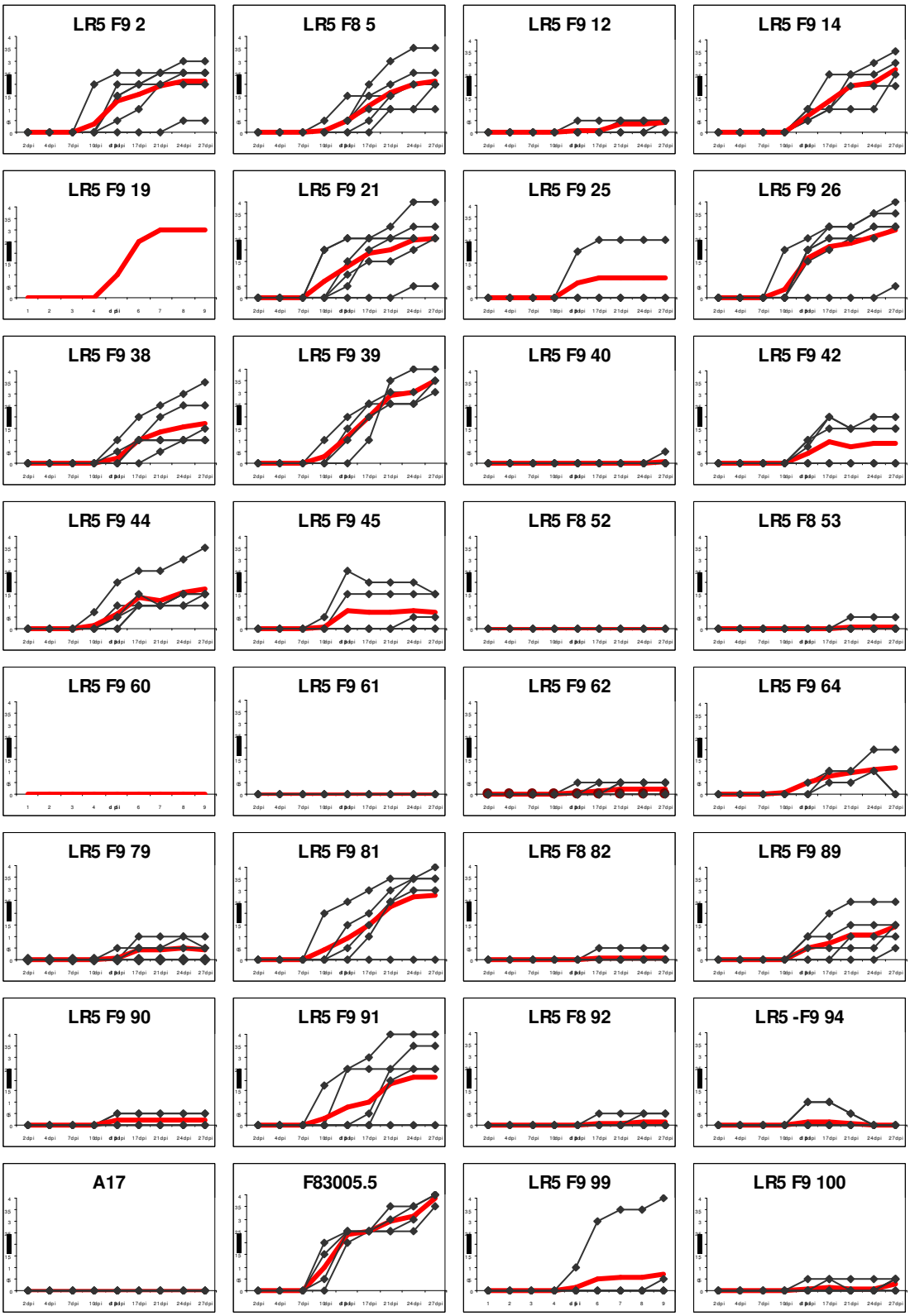
At this point, the Marker Assisted Selection, i.e. the selection of a quantitative character (the resistance to *V. albo-atrum*) based on the associated QTL, of the *M. truncatula* varieties will be possible.

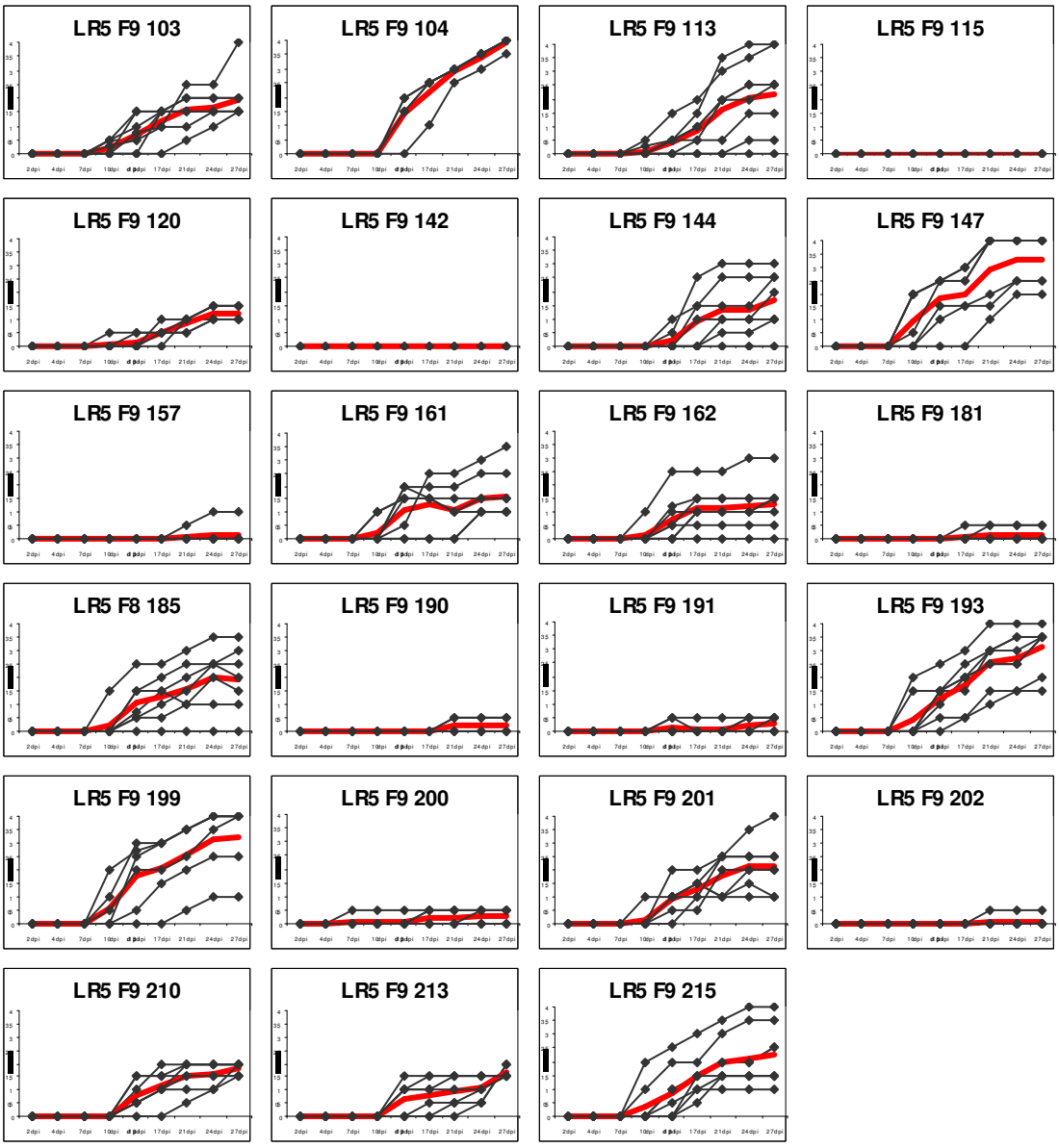
Concerning the *V. albo-atrum*-GFP strains, the study of the virulence might be repeated with a higher number of plants; furthermore, a parameter that describes the suffering of A17 in hydroponic culture should be made up. The microscope observations should be done periodically from the first hours post-inoculation. If there were other problems with the fluorescence of the GFP, the used promoter should be changed with another one independent from the metabolic activity of the fungus, as hypothesized above.

Meanwhile, the study of the colonization of *V. albo-atrum* on a cytological level could go ahead using the strains *C3D2 c'* and *C3A1 a'*.

ANNEXE I

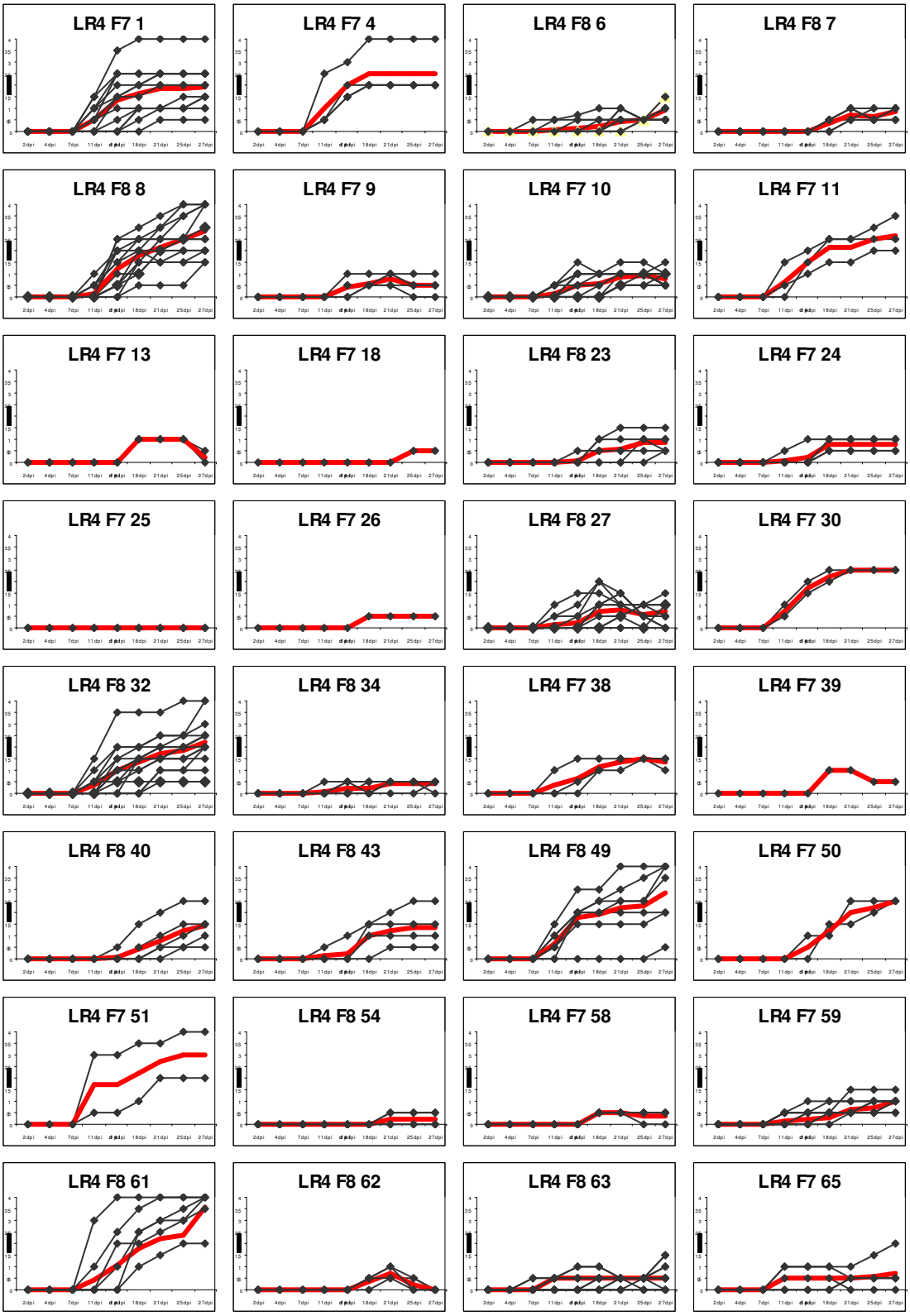
Variability amongst the lines of the LR5 population

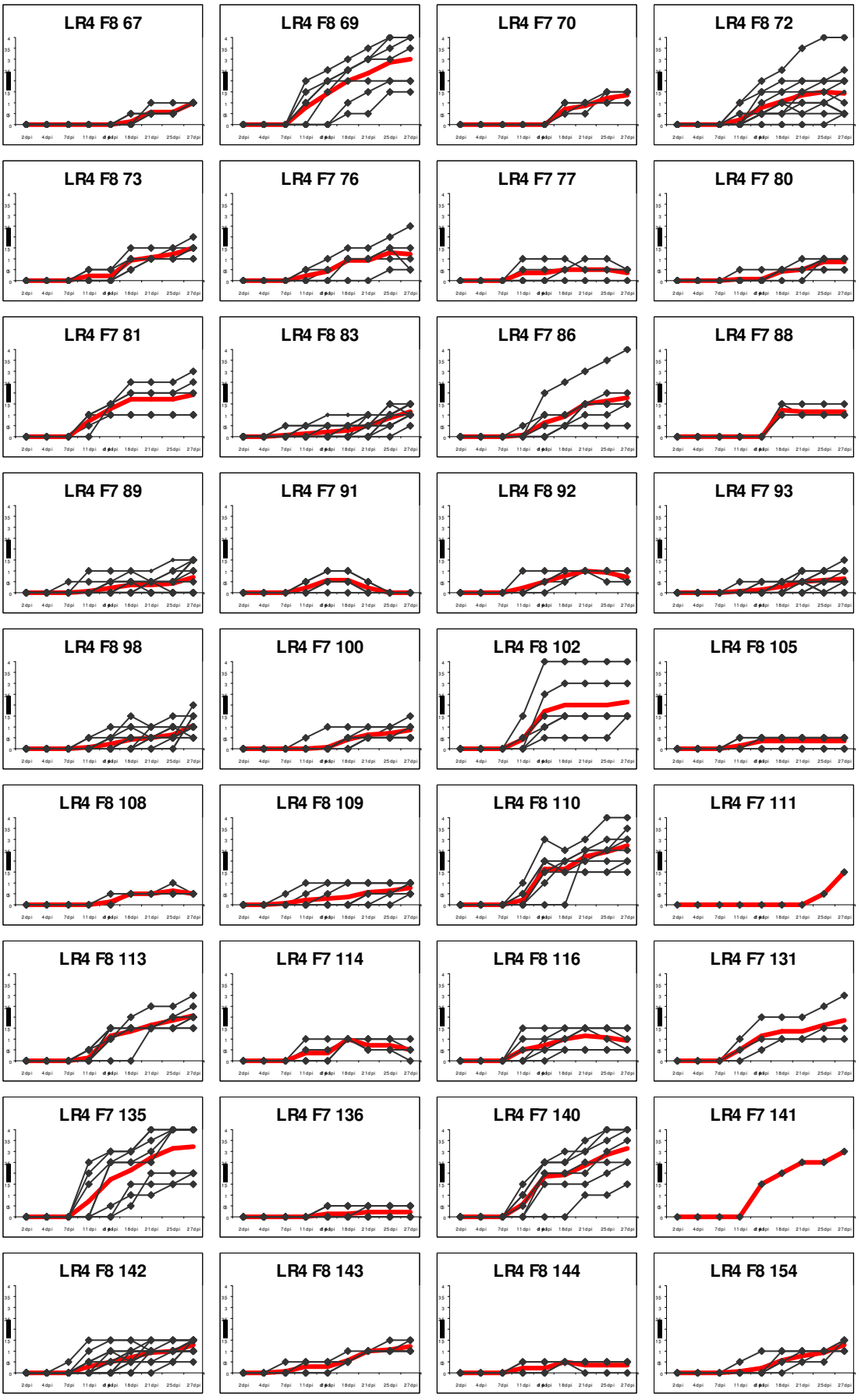



LR5 F9 103
LR5 F9 104
LR5 F9 113
LR5 F9 115
LR5 F9 120
LR5 F9 142
LR5 F9 144
LR5 F9 147
LR5 F9 157
LR5 F9 161
LR5 F9 162
LR5 F9 181
LR5 F8 185
LR5 F9 190
LR5 F9 191
LR5 F9 193
LR5 F9 199
LR5 F9 200
LR5 F9 201
LR5 F9 202
LR5 F9 210
LR5 F9 213
LR5 F9 215

ANNEXE II

Variability amongst the lines of the LR4 population




LR4 F8 67
LR4 F8 69
LR4 F7 70
LR4 F8 72
LR4 F8 73
LR4 F7 76
LR4 F7 77
LR4 F7 80
LR4 F7 81
LR4 F8 83
LR4 F7 86
LR4 F7 88
LR4 F7 89
LR4 F7 91
LR4 F8 92
LR4 F7 93
LR4 F8 98
LR4 F7 100
LR4 F8 102
LR4 F8 105
LR4 F8 108
LR4 F8 109
LR4 F8 110
LR4 F7 111
LR4 F8 113
LR4 F7 114
LR4 F8 116
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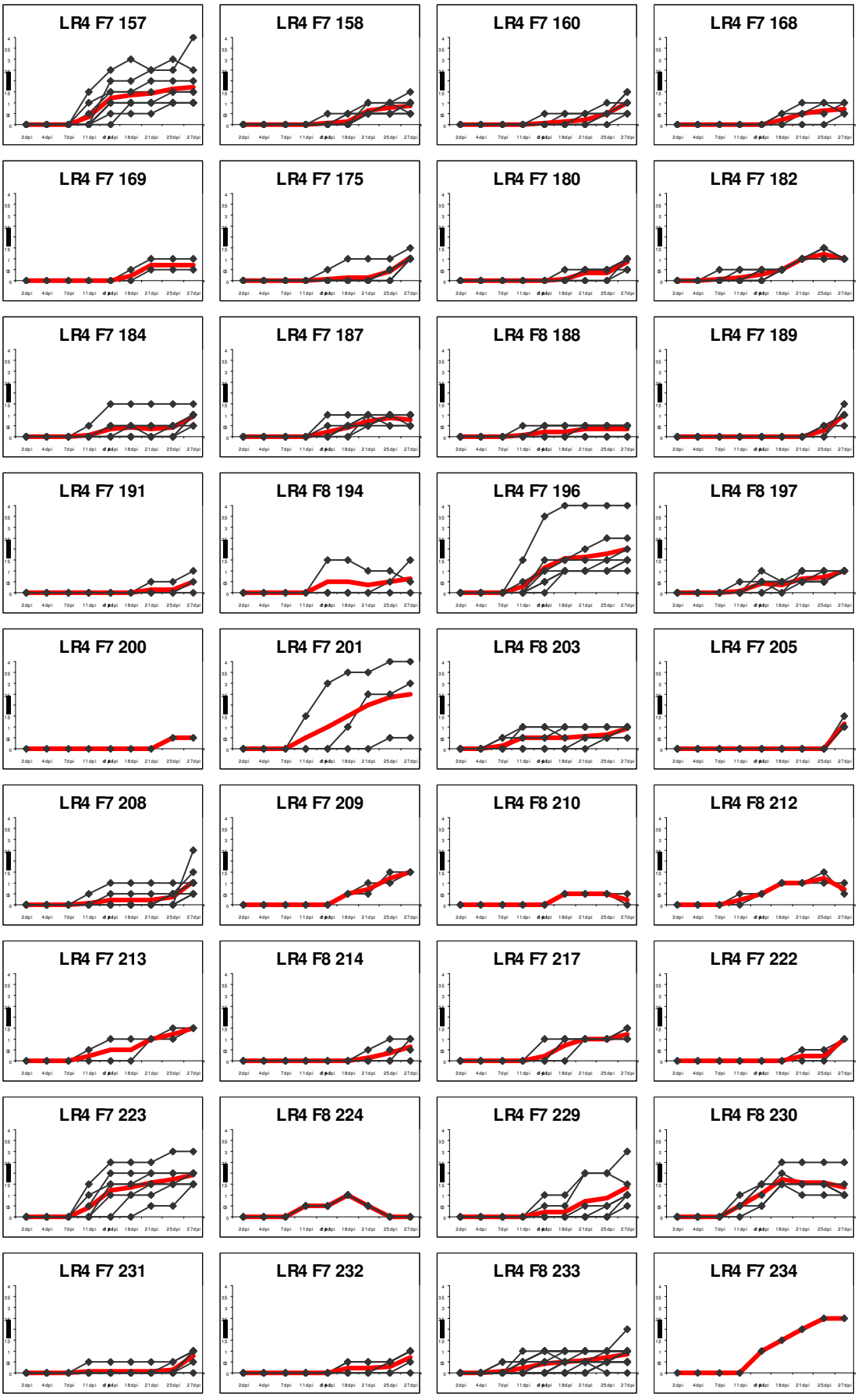
LR4 F8 142
LR4 F8 143
LR4 F8 144
LR4 F8 154


LR4 F7 157
LR4 F7 158
LR4 F7 160
LR4 F7 168
LR4 F7 169
LR4 F7 175
LR4 F7 180
LR4 F7 182
LR4 F7 184
LR4 F7 187
LR4 F8 188
LR4 F7 189
LR4 F7 191
LR4 F8 194
LR4 F7 196
LR4 F8 197
LR4 F7 200
LR4 F7 201
LR4 F8 203
LR4 F7 205
LR4 F7 208
LR4 F7 209
LR4 F8 210
LR4 F8 212
LR4 F7 213
LR4 F8 214
LR4 F7 217
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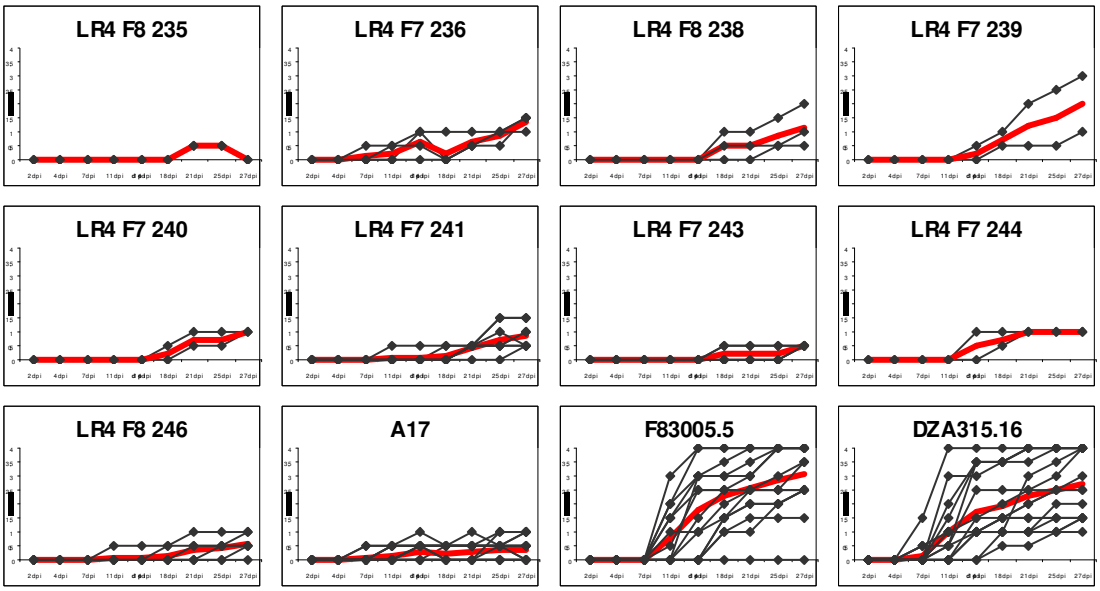
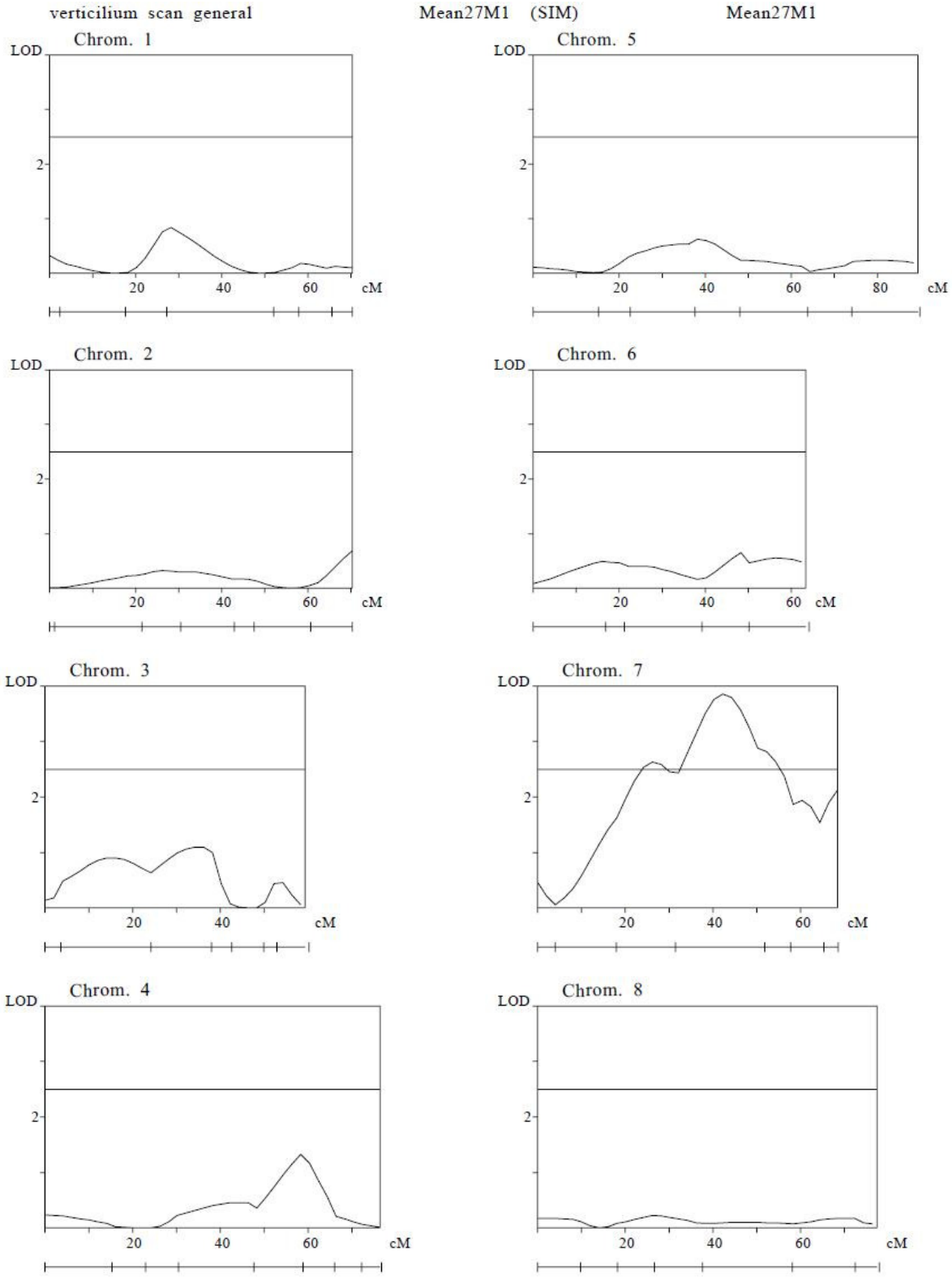
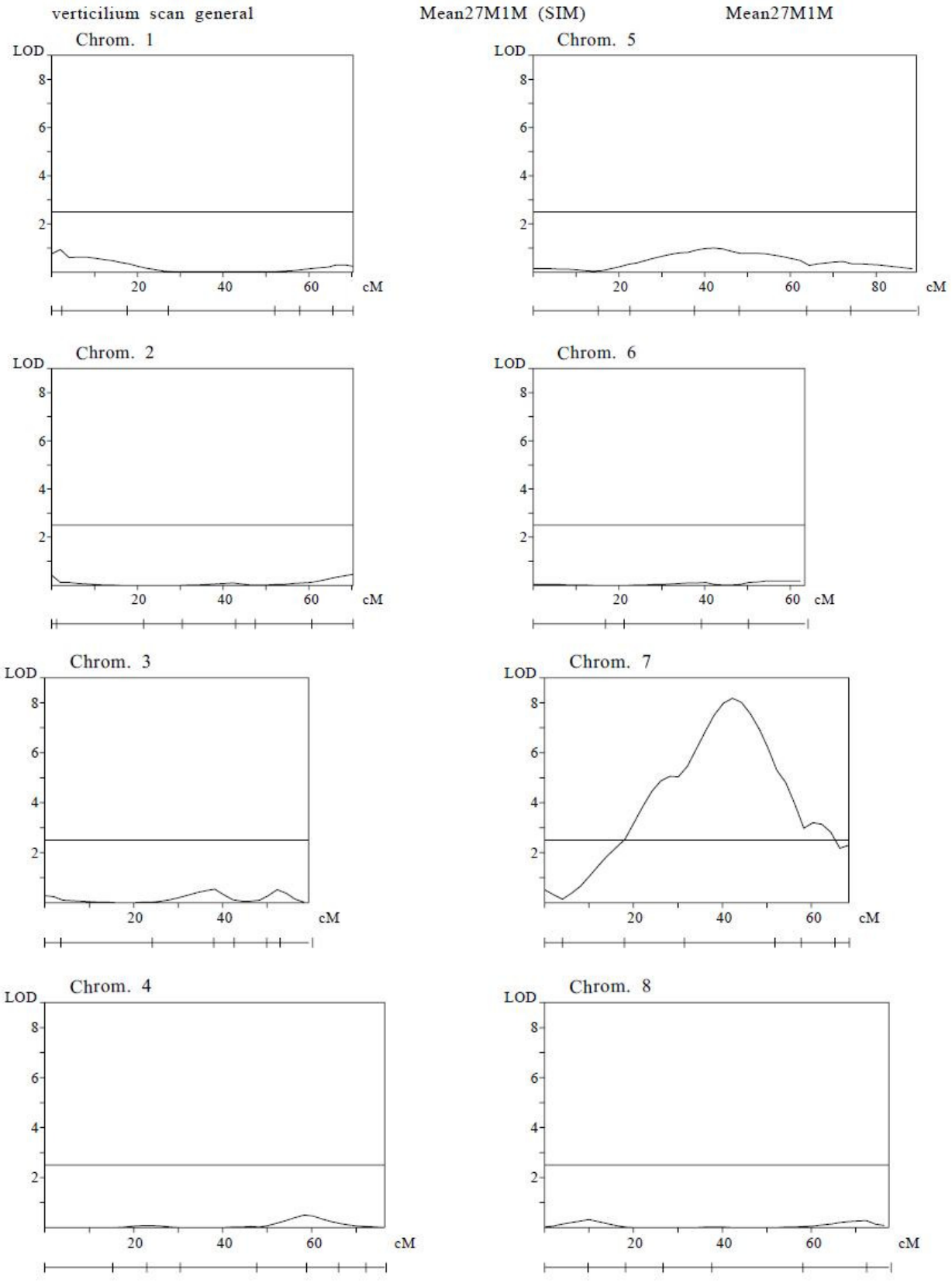
LR4 F7 231
LR4 F7 232
LR4 F8 233
LR4 F7 234


LR4 F8 235
LR4 F7 236
LR4 F8 238
LR4 F7 239
LR4 F7 240
LR4 F7 241
LR4 F7 243
LR4 F7 244
LR4 F8 246
A17
F83005.5
DZA315.16

ANNEXE III

Final simultaneous fit with all detected chromosomes for the first group of LR4 (Mean27M1) and for all the LR4 lines (Mean27M1M)




verticilium scan general
Mean27M1M (SIM)
Mean27M1M
Chrom. 1
Chrom. 5
Chrom. 2
Chrom. 6
Chrom. 3
Chrom. 7
Chrom. 4
Chrom. 8
LOD
cM

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http://www.ncbi.nlm.nih.gov.

www.genetics.org.

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